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NOTE

Lactuca spp. Seeds as a Bioindicator for the Toxicity of Gezira Tannery corporation wastewater

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Abstract

This study was conducted to establish the potential of lettuce (*Lactuca sativa L. var. buttercrunch*) seeds as a bioindicator (BI), or a biological tool for detecting the presence of some toxic materials used in tanning industry and determining their concentrations using the germination percentage as a parameter (indicator).

Samples of Gezira Tannery Corporation (GTC) wastewater (WW) were collected from both the mouth and the tail of the drainage stream.

Lettuce seeds (10/Petri dish, replicated 3x and each experiment was repeated 3x) were treated by GTC WW and other important tanning agents (chromium oxide , sodium sulfide, Preventol® WB) in solution using different concentrations of each and their mixture.

The bioassay experiment revealed that the seeds were intoxicated (*i.e.* reduced the germination percentage), when exposed to the WW. On exposure to several concentrations from each input, the concentrations that can be measured by this BI (i.e. sensitivity and reliability) are: chromium oxide from 0.1 to 3.25%, sodium sulfide from 0.19 to 1.5% and Preventol® WB from 18.75 to 150 ppm. Lower concentrations cannot be measured, and higher concentrations resulted in 100% inhibition. The IC_{50} was determined by probit analysis for the WW, mixture of the three inputs, chromium oxide alone, sodium sulfide alone and Preventol® WB alone were: 35.5, 14.5, 0.44, 0.45 and 0.005%, respectively. The slopes of the log-dose probability lines (Ld-P) showed that this BI response to all treatments was homogeneous (> 2) (tabulated X^2 ($df = n - 2$) at 5% = 0.172, 0.11, 0.064, 0.05 and 0.05). It is concluded that lettuce seeds satisfy almost all the required properties of the ideal BI.

Key Words: Bioindicater, Toxicity, Wastewater

Introduction

The leather tanning industry is a well-known for its severe negative impact on the environment. In this industry animal hides are transformed into leather in a succession of several complex stages, consuming high quantities of water and using large amounts of chemicals such as lime, sodium sulfide, ammonium sulfate, sodium chloride, bactericides,

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vegetable tannins, and chrome salts, *etc*. Tanneries wastewaters (WW) are mainly characterized by high salinity, high organic loading, and specific pollutants, such as sulfide and chromium (Song *et al.,* 2000). The tanning processes are the most contaminating to the environment mainly because of its high organic load and sulfide-content, as well as its content of inorganic salts of chloride, ammonia, chromium, and sulfate (Menendez and Diaz, 1998).

Pollution by tanneries did not receive enough attention in the Sudan. Large amount un-reacted (untreated) WW from Gezira Tannery Corporation (GTC), Wad Medani, Central Sudan, is discharged into the nearby areas, *viz*. the natural forest east of the tannery, which lies between the tannery and the blue Nile and Atra village. The objective of the present work is to determine the toxic effect of GTC wastewater and the individual inputs of this industry using the germination of *Lactuca sativa L. var. buttercrunch* (lettuce) seeds as a bioindicater, BI. Effect of different concentrations of this water and the inputs will also be investigated to provide data for the next series of the studies that will follow.

Materials and Methods

The experiments were conducted in the Plant Pathology Center laboratories, Faculty of Agricultural Sciences (FAS), University of Gezira (U of G). The wastewater (WW) samples were collected from the GTC, which contains some chemicals used in the industry as inputs.

Sampling: Samples of WW were collected from two locations: the outlet of the stream of WW and the bond inside the previous forest which is used as an evaporation site.

Lettuce Seeds (Lactuca sativa L. var. buttercrunch): Lettuce seeds were used as a bioindicator (BI), (Anon., 2006), to determine the toxicity of the WW of the GTC as a dose-response relationship bioassay. Moreover, the lettuce seeds were used to determine the effect of the most important or suspected toxic inputs of this industry.

The Bioassay Method: Lettuce seeds were soaked for 20 min in a 10% bleach solution (H_2O_2). They were rinsed five times to kill the fungal spores that might interfere with seed germination. A series of dilutions from the WW sample, chrome powder (oxide), sodium sulfides, Preventol WB® (fungicide) and mixture of (chrome powder oxide, sodium sulfides and Preventol WB®) were prepared. Two ml from each sample from all dilutions and all prepared compounds, in addition to 2 ml from distilled water (control) were placed in 7.5 cm in (*i.dia*.). Filter papers placed in 9 cm (*i.dia*.) Petri dishes. Ten lettuce seeds were added to each dish, spaced evenly on the filter paper. Then all dishes were placed in plastic bags and incubated in the dark at constant temperature (24.5°C) for 120 hr in the incubator model (R 216 GA). The germinated seeds in each dish were counted and compared with the control.

The following concentrations were used:

- In the case of the WW sample: 100 (as is), 50, 37.5, 25 and 12.5% were used.
- In the case of other chemicals: a series of dilutions were prepared and used based on the concentration of component input in GTC:

A) Mixture of (Cr2O3) 6.5%, (Na2S) 3%, Preventol WB 0.2%: 100 (as is), 50, 37.5, 25, 12.5 and 6.25 % were used.
B) Chromium Oxide (Cr2O3): 6.5, 3.25, 1.63, 0.81, 0.41, 0.20, 0.10, 0.05 and 0.03% were used.
C) Sodium Sulfide (Na2S): 3, 1.5, 0.75, 0.38, 0.19, 0.1and 0.05% were used.
D) Preventol WB: 0.20, O.10, 0.05, 0.03, 0.015, 0.0075, 0.00375 and 0.001875% were used.

Analysis of the Data: The data were subjected to probit analysis.

Table 1. The bioassay results of the GTC wastewater, mixture, $Cr2O_3$ (6.5%), Na2S (3%), and Preventol® WB (0.2%) on Seed Germination using the lettuce seed (*Lactuca sativa L. var. Buttercrunch*)

Treatment	IC50 (%)	IC90 (%)	Slope	Potential detectable concentration (% or ppm)
WW	35.5	72.4	4.17	12.5 - 100%
Mixture	14.5	31.6	3.4	6.25 - 50%
Chromium Oxide	0.44	1.61	2.26	0.1 - 3.25%
Sodium Sulfide	0.45	0.93	3.95	0.19 - 1.5 %
Preventol® WB	0.005	0.01	4.53	18.75 - 150 ppm

Results and Discussions

The results of the present work (Table 1) , confirm the results of Warner (2006) which reported that calcium chloride does have toxic effect on lettuce seeds and the present work showed that lettuce seeds can respond easily and almost accurately to the different concentrations of the tested pollutants up to specific concentrations. Some pollutants were even more potent (more inhibitors) than the others. The slopes of the Ld-P lines reflected the homogeneity or heterogeneity of the seed population to each pollutant.

Conclusion

These effluents, according to the results of the present work, proved to be toxic to lettuce seeds, *Lactuca sativa L. var. buttercrunch*, which were used as a bioindicator to determine the presence of toxicants/pollutants, and to investigate the acute toxicity, in terms of percent germination inhibition, for the wastewater, chromium oxide, sodium sulphid and Preventol WB (fungicide), and their mixture. The mixture of three compounds was more potent than the effluents. The seeds also proved to be very sensitive regarding the dose-response relationship of each of these pollutants, the WW and the mixture.

Therefore, these seeds can be used to indicate/monitor contaminant exposure, can also help identifying mechanisms of toxicity, can provide early warning of impending environmental damage, and can also provide early indications of environmental recovery (remediation). Moreover, it is important in linking cause (stressor/pollutant) to ecologically relevant effects, and can be incorporated into ecological risk assessment

References

Anonymous (2006). *Lettuce Seed Bioassay*, Cornell University and Penn State University.

Environmental Inquiry. Lettuce seed Bioassays. htm.

Menendez, C., and Diaz, M. (1998). Tannery Waste Water Treatment (in Spanish). *Technologia de Agua* 183: 27–32 (Seen as an abstract).

Richard, H., and Ivanildo, H. (1997). Water Pollution Control—A guide to the use of water quality management principles, *WHO/ UNEP, ISBN 0419 2291O8*, p: 34.

Song, Z., and Williams, C. J, (2000). Sedimentation of Tannery Wastewaters-Water *Res* 34: 2171–2176

Warner, D (2006). Lettuce seed Dose/response Bioassay, Cornell University. *Environmental inquiry. Lettuce seed Bioassays.htm.*

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NOTE

Oxytetracycline residues in bovine carcasses slaughtered at Mansoura Abattoir, Egypt

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Abstract

Oxytetracycline residues were examined in 600 samples (200 each of muscles, livers and kidneys) collected randomly from bovine carcasses slaughtered at Mansoura abattoir in Dakahlia Province, Egypt. A microbial inhibition test using *Bacillus subtilis* ATCC- 6633 was employed to screen the obtained samples for antibiotic residues in meat. The results showed that 2% of samples were positive. Oxytetracycline residues exceeded the maximum residue limits (MRLs) in 1.33% of the examined samples. Thus, regulatory authorities should insure proper withdrawal period before slaughtering of the animals (28 days for oxytetracycline). Public health importance was discussed.

Key Words: Oxytetracyclines, bovine, Egypt

The use of antimicrobial agents in historical perspective has been increased soon after the introduction of antimicrobial agents for therapy of bacterial infections in human and animals. The growth promoting effect of antimicrobial agents was observed, and since the beginning of the 1950, antimicrobial agents have been included in feed for food animals as a way to improve growth and reduce production costs. The available data for different countries show that the use of antimicrobial agents for growth promotion normally equals or exceeds the usage of antimicrobial agents for therapy of food animals. The occurrence of resistance to antimicrobial agents used for growth promotion indicates that resistance will also emerge following the introduction of antimicrobials for growth promotion[1]. Today, antimicrobial drugs are used to control, prevent and treat infection and to enhance animal growth and feed efficiency[3]. Tetracyclines among the first of the antibiotics to become available 50 years ago, are still widely used. Tetracyclines have bacteriostatic activity against a wide variety of pathogens that are responsible for many common and some exotic infections. These antibiotics are widely used for the treatment of bovine mastitis and are added to subtherapeutic levels to cattle feed for prophylaxis[5,13]. In human medicine, drug allergy is a well established side effect of the therapeutic use of antibiotics, especially the beta-lactams. Side effects caused by macrolides are uncommon and only a very few of these seem to be caused by allergic mechanisms. Clinically, drug allergy

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is characterized by a spectrum of reactions ranging from mild skin rashes to angio-oedema or life threatening anaphylaxis. Concern has been expressed that antibiotic residues in meat and other foods might be responsible for similar hypersensitivity reaction in a small number of individuals[10]. Therefore, the aim of this study was to determine the antibiotic residues in cattle carcasses qualitatively using *Bacillus subtilis* screening method and to quantitatively estimate oxytetracycline residues using high performance liquid chromatography (HPLC).

Thus, a total of 600 random fresh cattle meat samples include muscle, liver and kidney, 200 for each from 200 slaughtered male animals in Mansoura abattoir, Dakahlia Province, Egypt. 50–100 gm of labeled samples (muscle- liver-kidney) were obtained from each carcass which were wrapped in polyethylene bags and were put in cool boxes with dry ice or freezer packs at 4°C. The samples were subsequently transported directly under a complete aseptic condition and rapidly without undue delay to our laboratory. The first screening of the collected samples for presence of antibiotic residues was done using the four plate method[3] using nutrient agar plates seeded with *Bacillus subtilis* (ATCC-6633) at pH 6 and pH 8. In brief, a cylinder of each tissue sample was cut into four fat-free slices, two of which were placed on each pH agar plates. The inoculated plates were incubated for 18–24 h at 30 ± 1°C. The width of each inhibition zone was then measured from the edge of the sample to the edge of the inhibition zone. High Performance liquid chromatography (HPLC) analysis for positive was used for determination of oxytetracycline level[8,11]. Briefly, two grams of each organ to be analyzed were weighed, cut into very small pieces and subsequently ground into fine powder using Sartorius mincer. This was then homogenized in a blender for 2 min and then 0.1 gm citric acid was added. To this mixture, 1 ml nitric acid (30%), 4 ml methanol and 1 ml deionized water were added, respectively. The suspension with solid particles was put in a vortex for mixing, kept in an ultrasonic bath for 15 min and centrifuged for 10 min at 5300 rpm. After filtering through a 0.45 μm nylon filter, 20 μl of solution was injected into HPLC for analysis. A mobile phase of methanol and formic acid 0.1% using a gradient method with a flow rate of 1.5 ml/min at 25°C was used. The separation was done on hypersil gold C18 (10 μm, 100 × 4.6 mm) column with mobile phase. Detection was performed with PDA detector set at 350 nm wave length. Quantification of residues in samples was obtained and calculated from areas under curves extrapolated automatically by the software (Chromo Quest 5). Calibration curve was prepared by using concentrations of 0.01, 0.1, 0.5, 1.25, 2.5, 5, 10, 20, 50 mg/L of oxytetracycline in eluent. The detection limit for oxytetracycline was 0.01 ppm while the retention time was 3.7 min.

The results of the four plate test for antibiotic screening showed that 12 out of 600 examined samples were positive for the antibiotic residues with a percentage of 2%. The percentage of the antibiotic residues in slaughtered cattle in Mansura slaughter house was much lower was much lower than slaughtered cattle in Ghanawa slaughter house in Khartoum state, Sudan[4], as the positive cases represented 33% of the slaughtered cattle. The tissue distribution of the positive cases was 3, 6 and 3 cases in the muscles, liver and kidney respectively. The diameter of the zone of inhibition ranged between 4–8 mm (Data not shown). These results are consistent with those recorded in other investigations[7,9,10]. Oxytetracycline was selected to measure its residual levels due to the frequent usage of it in the Egyptian farms. Quantitative measurement of oxytetracycline residues in the microbiologically positive samples for antibiotic residues showed that 8 out of the 12 positive samples contained oxytetracycline residues (Table 1). Five samples had oxytetracycline residues than the recommended permissible limits[6]. Presence of high levels of oxytetracycline residues in liver and kidney compared with the

Table 1. Concentration of oxytetracycline residues (μg/kg) in the microbiologically positive meat samples for antibiotic residues

Organ	Concentration (μg/kg wet weight)	Maximum permissible limits (μg/kg)	Concentration compared with MPL
Kidney	11516.17	1200	Above
Liver	31516.24	600	Above
Liver	16205.12	600	Above
Kidney	10925.07	1200	Above
Muscle	164.596	200	Below
Liver	45.764	600	Below
Liver	256.052	600	Below
Muscle	9405.964	200	Above

N = 200 for each of muscle, liver and kidney
MPL means Maximum Permissible Limits according to FAO (1999)

muscle is considered reasonable as both organs are contributed in drug metabolism and clearance. Similar findings were recorded in United Kingdom, Finland, Sudan, Kenya and Iran[2,4,9,12]. The presence of oxytetracycline residues in the meat destined for human consumption at high levels could be as a result of the indiscriminate use and misuse of veterinary drugs as commonly practiced among livestock producers and marketers without observing withdrawal period prior to slaughter, consumers are predisposed to health hazards.

In conclusion, Meat producers should observe carefully the drug withdrawal times before slaughtering as that may lead to high residual levels of antibiotics and render these meats unsuitable for human consumption.

References

1) Aarestrup, F. M. 2000. Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. *APMIS Suppl.,* **101**: 1–48.
2) Abbasi, M. M., Rashidi, M. R., Javadi, A., Amirkhiz, M. B., Mirmahdavi, S. Zabihi, M. 2009. Levels of tetracycline residues in cattle meat, liver and kidney from a slaughterhouse in Tabriz, Iran. *Turk. J. Vet. Anim. Sci.,* **33**(4): 345–349.
3) Aerts, M. M. L., Hogenboom, A. C. and Brinkman, U. A. 1995. Analytical strategies for the screening of veterinary drugs and their residues in edible products. *J. Chromatogr.,* **667**: 1–20.
4) Alla, M. B. W., Mohamed, T. E. and Abdelgadir, A. E. 2011. Detection of antibiotics residues in beef in Ghanawa Slaughterhouse, Khartoum State, Sudan. *Afr. J. Food Sci.,* **5**(10) 574–580.
5) Dewdney, J. M., Maes, L., Raynaud, J. P., Blanc, F., Scheid, J. P., Jackson, T., Lens, S. and Verschueren, C. 1991. Risk assessment of antibiotic residues of beta-lactams and macrolides in food products with regard to their immuno-allergic potential. *Food Chem. Toxicol.*, **29**(7): 477–483.
6) Food Agricultural Organization (FAO) 1999. Residues of some veterinary drugs in animals and foods. Food and nutrition paper 41/3: 97–119.
7) Masztis, P. S. 1984. Antibiotic residues testing in a beef slaughterhouse. *Can. Vet. J.,* **25**: 329–330.
8) Moats, W. A. 1986. Determination of tetracycline antibiotics in tissues and blood serum of cattle and swine by HPLC. *J. chromatogr.,* **358**: 253–259.
9) Muriuki, F. K., Ogara, W. O., Njeruh, F. M. and Mitema, E. S. 2001. Tetracycline residue levels in cattle meat from Nairboi slaughterhouse in Kenya. *J. Vet. Sci.,* **2**(2): 97–101.
10) Myllyniemi, A. L., Rannikko, R., Lindfors, E., Niemi, A. and Backman, C. 2000. Microbiological and chemical detection of incurred pencillin G, oxytetracycline,

enrofloxacin and ciprofloxacin in bovine and porcine tissues. *Food Addit. Contam.*, **17**(12): 991–1000.
11) Senyuva, H., Ozden, T. and Sarica, D. Y. 2000. High-performance liquid chromatographic determination of oxytetracycline residue in cured meat products. *Turk. J. Chem.*, **24**: 395–400.
12) Smither, S., Lott, A. F., Dalziel, R. W. and Ostler, D. C. 1980. Antibiotic residues in meat in the United Kingdom, an assessment of specific tests to detect and identify antibiotic residue. *J. Hyg. (Lond.).,* **85**(3): 359–369.
13) Tollfeson, L. and Miller, M. A. 2000. Antibiotic use in food animals: Controlling the human health impact. *J. AOAC. Int.,* 83: 245–256.

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NOTE

Biological responses of xenobiotic metabolizing enzymes to lead exposure in cultured H4IIE rat cells

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Abstract

This study was undertaken to investigate the constitutive response of xenobiotic metabolizing enzymes (XMEs) to lead (Pb^{2+}) exposure in cultured rat liver (H4IIE) cell lines. Phase I enzymes such as CYP1A1 and CYP1A2 had mRNA expressions that were slightly induced after exposure to low concentrations of Pb^{2+}; however, under higher concentrations of Pb^{2+}, the mRNA expressions of CYP1A1 and CYP1A2 were significantly down-regulated. These effects were in correspondence with AhR mRNA expression. Phase II enzymes had mRNA expressions that were reduced upon exposure to Pb^{2+}. Metallothionein mRNA expression was induced after treatment with Pb^{2+} in a dose-dependent trend. In conclusion, Phase I and II enzymes were significantly modulated upon lead exposure indicating some toxicological implications for lead exposure in cultured H4IIE cells.

Key Words: lead, xenobiotic metabolizing enzymes, rat

Lead (Pb^{2+}) is a common environmental contaminant, which is released from sources such as municipal and industrial waste incineration and combustion[10]. Pb^{2+} is widely used in foundries, mining, manufacturing industries, and electrical instruments and exists in the environment either in a solid form as particulates or in a vapor form[2]. Pb^{2+} is a ubiquitous, highly toxic, non-essential element that neither created nor biodegradable[1]. Frequently persistent occurrences and accumulations of heavy metals such as Pb^{2+} in the environment and their potential exposure to living organisms has biological consequences, and such effects involve the xenobiotic metabolizing enzyme (XME) system.

Cytochrome P450s (CYP) constitutes a major family of XMEs that transform foreign chemicals to non-toxic or carcinogenic metabolites. Among these, CYP1A1 and 1A2 are of major interest because of their role in metabolizing procarcinogenic and environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs)[4]. Most compounds undergo phase II conjugation

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reactions that involve glucoronidation via uridine diphosphate-glucuronosyl transferases (UGTs), glutathione conjugation via glutathione-S-transferases (GST), sulfation via sulfotransferases (SULT), and NAD(P)H: quinine redox via oxidoreductase 1 (NQO1).

Regulation and expression of these XMEs are mediated through a cytosolic receptor named aryl hydrocarbon receptor (AhR). Mechanistically, AhR is located in the cytoplasm in an inactive form bound to other proteins, such as heat shock protein 90 (HSP90), forming an inactive complex. AhR is activated upon binding with AhR ligand-like dioxins, leading to translocation of AhR into the nucleus. In the nucleus, the ligand-receptor complex dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT), which binds to xenobiotic-responsive elements (XRE) located in the promoter region of each AhR-regulated gene, and this results in transcription and ultimately protein translation[8].

Little information is available about the effects of heavy metals, particularly Pb^{2+}, on the regulation and expression of AhR-regulated genes.

Metallothionein (MT) is a ubiquitous, cysteine-rich, metal binding protein. It has been assumed that MT plays a role in the metabolism and detoxification of heavy metals such as cadmium and mercury. Moreover, MT is highly involved in the protection of tissues against various forms of oxidative injury, including radiation, lipid peroxidation, and oxidative stress produced by heavy metals[9]. Thus, the induction of MT gene expression is considered as a valuable marker for heavy metal exposure (particularly with respect to cadmium, mercury, and zinc). However, the relationship between lead exposure and MT gene expression is less well-known. Thus, this study was undertaken to investigate the biological responses of XMEs, including CYP1A1, 1A2, UGT1A6, GST1A, NQO1, and SULT1C1 to lead exposure in cultured rat H4IIE cells. In addition, the effects of Pb^{2+} on AhR and MT-1 mRNA expression were also examined.

All experiments were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. H4IIE rat hepatoma cells obtained from the American Type Culture Collection (Manassas, VA), were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St, Louis, MO) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified incubator with 5% CO_2. Cells were seeded in 60 mm collagen-coated dishes, sub-cultured twice a week, and subsequently grown to 80–90% confluence. Cells were exposed to lead acetate (Pb^{2+}) (0–10 μM) in serum-free medium for 24 h. After exposure of the cells to Pb^{2+}, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). The cell viability was assayed using the CCK-8 assay (Sigma-Aldrich) by measuring the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye. Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, USA). The RNA quality was estimated by the 260/280 nm and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis. The cDNA was synthesized according to our previous report[5]. In brief, a mixture of 5 μg of total RNA and 0.5 ng of oligo dT primer in a total volume of 24 μl of sterilized ultra-pure water was incubated at 70°C for 10 min and then removed from the thermal cycler, and the volume was increased to 40 μl with a mixture of 4 μl (5X) RT-buffer (Toyobo Co., Ltd, Osaka, Japan), 8 μl 10 mM dNTP, 2 μl water, and 2 μl reverse transcriptase (Toyobo Co., Ltd). The mixture was then re-incubated in the thermal cycler at 42°C for 45 min and at 90°C for 10 min to prepare the cDNA. Quantitative real-time PCR for rat mRNA levels was performed using TaqMan Gene Expression Assays (Applied

Biosystems, CA, USA) and measured by StepOne™ Real-Time PCR System (Applied Biosystems). The primer and probe sets for each specific gene were as follows: Rn00487218_m1 (CYP1A1), Rn00561082_m1 (CYP1A2), Rn00565750_m1 (AhR), Rn00756113_AH (UGT1A6), Rn00755117_A1 (GSTA1), Rn00566528_m1 (NQO1), Rn00581955_m1 (SULT1C1), and Rn99999916_s1 (GAPDH). The reaction was performed for 40 cycles: initial activation at 95°C for 20 sec, denaturation at 95°C for 1 sec, annealing and extension at 60°C for 20 sec. The measurements of specific enzyme and receptor genes and GAPDH were performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of GAPDH and was calculated relative to that of controls using the comparative threshold cycle (Ct) method. In addition, we used the following specific primers to measure the expression of the metallothionein-1 (MT-1) gene: forward primer 5'-caccgttgctccagattcac-3', reverse 5'-aggagcagcagctcttcttg-3' (Accession number: NG_006919.3); and GAPDH, forward 5'-gtcttcacc accacggagaaggc-3'; reverse 5'-atgccagtgagcttcccgtt cagc-3' (Accession number: XR086293.2). To measure the expression levels of MT-1 and GAPDH genes, SYBR Green Real-Time PCR Master Mix (Finnzyme, Espoo, Finland) was used. The conditions of the PCR reaction were as follows: the initial cycle was 50°C for 2 min and 95°C for 10 min, and then 45 cycles were performed at 95°C for 15 sec and 60°C for 1 min. GAPDH was used for the normalization in the comparative Ct method. Statistical significance was evaluated using the Tukey-Kramer HSD test (JMP statistical package, SAS Institute Inc., Cary, NC, USA). A P-value < 0.05 was considered to be significant.

Exposure to heavy metals such as Pb^{2+} is a common environmental problem with biological consequences. However, there have been few studies that have investigated the constitutive response of XMEs to Pb^{2+} exposure in rats, especially in the absence of AhR ligands. Thus, this study was undertaken to investigate the constitutive response of XMEs to Pb^{2+} exposure in cultured rat liver (H4IIE) cell lines.

Exposure of H4IIE cells to different concentrations of Pb^{2+} did not alter the cell viability (data not shown). Interestingly, both CYP1A1 and CYP1A2 mRNA expressions were induced after exposure to low concentrations (0.25 μM) of Pb^{2+}. This induction was markedly decreased under high concentrations of Pb^{2+} (5–10 μM) (Fig. 1 A, B). These results corresponded highly with AhR mRNA expression. Pb^{2+} treatment induced AhR mRNA expression under low concentrations (0.25 μM) and down-regulated AhR mRNA expression under high Pb^{2+} concentrations (5–10 μM) (Fig. 2A).

Regulation of the CYP1A subfamily (1A1 and 1A2) has been shown to involve the activation of the AhR-dependent pathway by direct binding of AhR-ligands to the receptor, AhR[11]. Interestingly, Pb^{2+} does not have structural properties and similarities to classical AhR ligands, suggesting that it could be a novel non-classical inducer of AhR that also induces CYP1A expression without binding to AhR. The inhibition of CYP1As and AhR expression by high concentrations of Pb^{2+} might be explained by the induction of oxidative stress. Heme oxygenase-1 (HO-1) mRNA, which is used as a biomarker for oxidative stress, was significantly induced in murine Hepa1c1c7 cells exposed to lead, copper, and mercury[8].

Phase II enzymes (UGT1A6, GST1A, NQO1, and SULT1C1) showed higher sensitivity to lead exposure as they were significantly reduced upon exposure to even low concentrations of Pb^{2+}, especially in the case of GST1A and SULT1C2 (Figs. 1C, D, E, and F). These results are in agreement with another report[8] demonstrating that the induced GST- and NQO1-dependent activities in the murine Hepa1c1c7 cell lines exposed to AhR ligands, such as 3-methylcholanthrene and benzo[a]pyrene, were significantly reduced after co-exposure with increasing doses of Pb^{2+}. The clear down-regulation of phase II enzymes, especially under

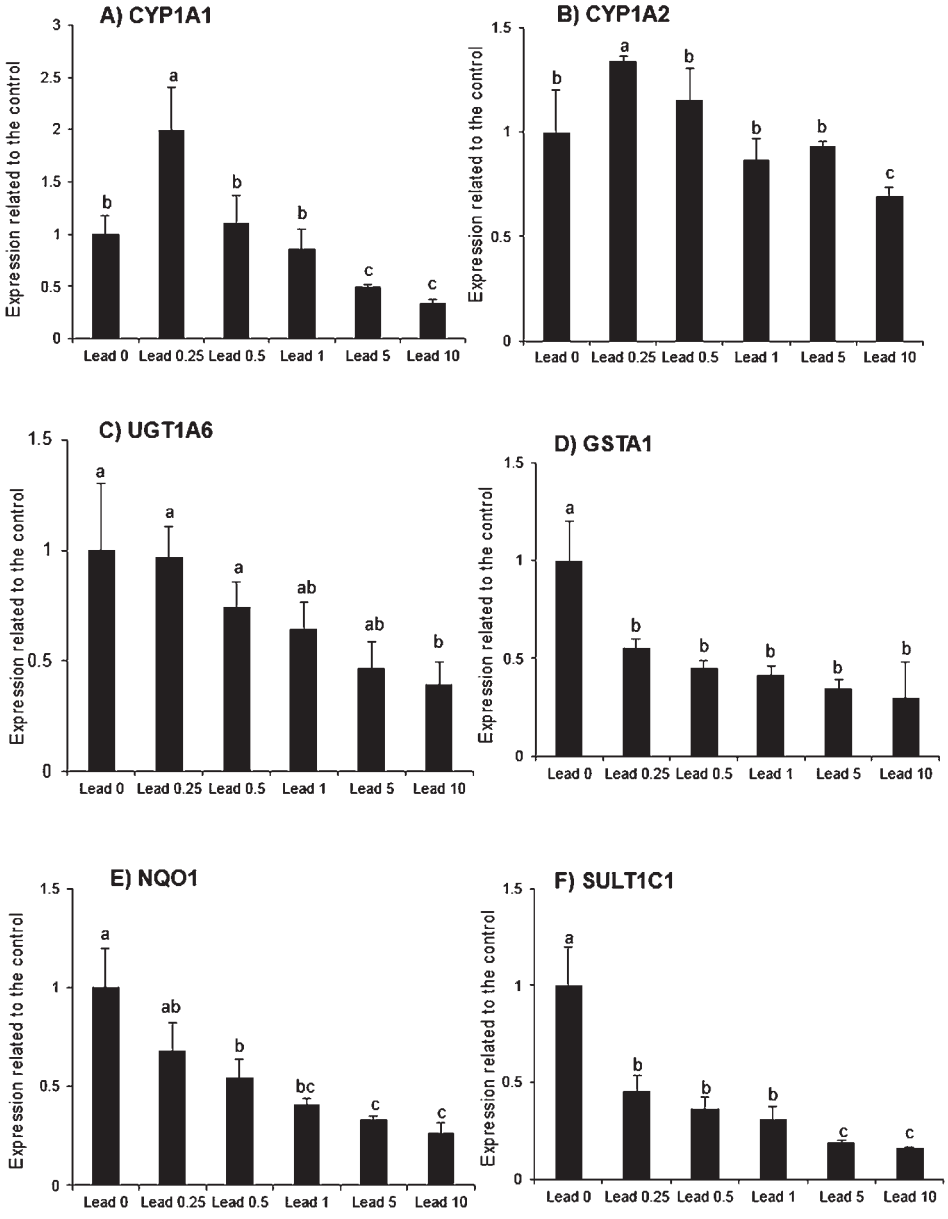

Fig. 1. Phase I and II mRNA expressions in H4IIE rat cells treated with lead. The effect of Pb^{2+} treatments on: A) CYP1A1, B) CYP1A2, C) UGT1A6, D) GST1A, E) NQO1, F) SULT1C1 mRNA expressions in H4IIE rat cells using real-time RT-PCR. The cDNA samples were amplified as described in the text. The amount of each enzyme was normalized to the corresponding amount of GAPDH and presented relative to the cells treated with water. Each treatment is represented by five plates. Data are presented as the mean ± standard deviation (SD). Identical letters were not significantly different from each other. $P < 0.05$.

increasing doses of lead, may be attributed to metal-mediated oxidative stress, which includes the production of reactive oxygen species (ROS) and lipid peroxidation[7]. This interference with phase I and II enzymes expression may have some toxicological implications in H4IIE cells, particularly in the case of co-exposure to lead and other xenobiotics.

Few studies have examined MT chelating lead because cadmium is considered the principal MT inductor. Nevertheless, the results reported from this study demonstrated that MT in H4IIE rat cells was also affected by exposure to Pb^{2+} in a dose-dependent trend (Fig. 2B). The same

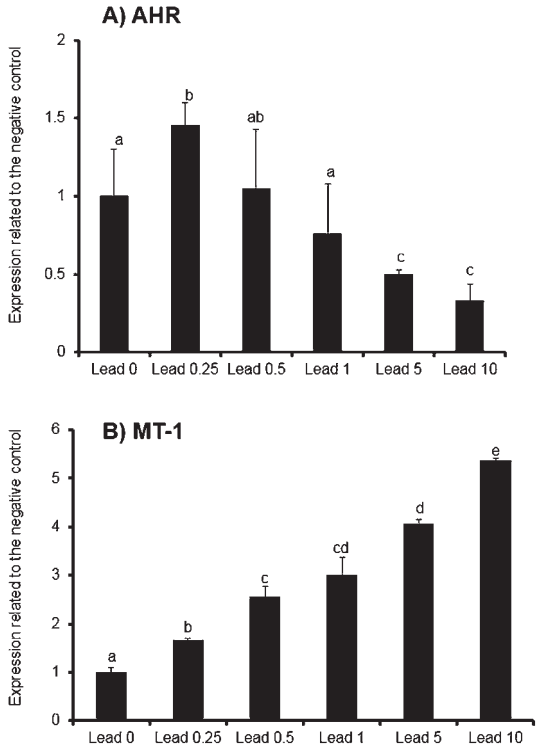

Fig. 2. AhR and MT-1 mRNA expressions in H4-II-E rat cells treated with lead. The effects of Pb^{+2} treatments on: A) AhR, and B) MT-1 mRNA expressions in H4-II-E rat cells using real-time RT-PCR. The cDNA samples were amplified as described in the Materials and Methods section. The amount of each enzyme was normalized to the corresponding amount of GAPDH and presented relative to the cells treated with water. Each treatment is represented by five plates. Data are presented as the mean $\pm$ SD. Identical letters were not significantly different from each other. $P < 0.05$.

physiological trend was highlighted in other studies where dose-response relationships between injected cadmium and MT in toadfish liver were investigated.[3] Another report published by our laboratory confirmed that MT is also induced in cattle blood cells after lead treatment in a dose-dependent fashion.[6] Thus, MT could be used as a biomarker for lead exposure.

In conclusion, this study highlighted the effects of lead on XMEs in rat H4IIE cells. Phase I and II enzymes were significantly modulated upon lead exposure, indicating some toxicological implications for lead on XMEs. This modulation may be attributed to mechanistic pathways. Furthermore, MT is induced after lead treatment, suggesting that it could be used as a biological biomarker for lead exposure in rats. Future approaches are needed to elucidate the exact mechanisms mediating these effects of lead on XMEs in rats.

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References

1. Barbier, O., Jacquillet, G., Tauc, M., Cougnon, M. and Poujeol, P. 2005. Effect of heavy metals on, and handling by, the kidney. *Nephron Physiol*, **99** (4): 105–10.
2. Brodkin, E., Copes, R., Mattman, A., Kennedy, J., Kling, R. and Yassi, A. 2007. Lead and mercury exposures: interpretation and action. *CMAJ*, **176** (1): 59–63.
3. Campana, O., Sarasquete, C. and Blasco, J. 2003. Effect of lead on ALA-D activity, metallothionein levels, and lipid peroxidation in blood, kidney, and liver of the toadfish Halobatrachus didactylus. *Ecotoxicol. Environ. Safety*, **55**: 116–125.
4. Darwish, W. S., Ikenaka, Y., Eldaly, E. and Ishizuka, M. 2010. Mutagenic activation and detoxification of benzo[*a*]pyrene *in vitro* by hepatic cytochrome P450 1A1 and phase II enzymes in three meat-producing animals. *Food Chem. Toxicol.*, **48**: 2526–2531.
5. Darwish, W. S., Ikenaka, Y., Ohno, M., Eldaly, E. And Ishizuka, M. 2010. Carotenoids as regulators for inter-species difference in Cytochrome P450 1A expression and activity in food producing animals and rats. *Food Chem. Toxicol.*, **48**: 3201–3208.
6. Ikenaka, Y., Nakayama, S. M., Muroya, T.,

Yabe, J., Konnai, S., Darwish, W. S., Muzandu, K., Choongo, K., Mainda, G., Teraoka, H., Umemura, T. and Ishizuka, M. 2012. Effects of environmental lead contamination on cattle in a lead/zinc mining area: Changes in cattle immune systems on exposure to lead in vivo and in vitro. *Environ. Toxicol. Chem.*, **31** (10): 2300–5.

7. Kaliman, P. A., Nikitchenko, I. V., Sokol, O. A. and Strel'chenko, E. V. 2001. Regulation of heme oxygenase activity in rat liver during oxidative stress induced by cobalt chloride and Hg^{+2} chloride. *Biochem.,* **66**: 77–82.
8. Korashy, H. and El-Kadi, A. 2004. Differential effects of mercury, lead, and copper on the constitutive and inducible expression of aryl hydrocarbon receptor (AHR)-regulated genes in cultured hepatoma Hepa 1c1c7 cells. *Toxicol.,* **201**: 153–172.
9. Sato, M. and Kondoh, M. 2002. Recent studies on metallothionein: Protection against toxicity of heavy metals and oxygen free radicals. *Tohoku J. Exp. Med.,* **196**: 9–22.
10. Vakharia, D. D., Liu, N., Pause, R., Fasco, M., Bessette, E., Zhang, Q. Y, and Kaminsky, L. S. 2001. Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures. *Toxicol Appl Pharmacol.,* **170** (2): 93–103.
11. Whitlock, J. P. 1999. Induction of cytochrome P4501A1. *Annu. Rev. Pharacol. Toxicol.,* **39**: 103–125.

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NOTE

Heavy metal residues in canned fishes in Egypt

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Abstract

A total of 75 random canned fish samples, 25 each of canned (canned tuna, sardine and mackerel) during 2009, were collected from Zagazig Markets for determination of lead, cadmium, zinc, copper and tin residues using atomic absorption spectrophotometer. The obtained results revealed that the mean values of the lead residues in the examined canned tuna, sardine and mackerel were 0.127 ± 0.02, 0.013 ± 0.004 and 0.023 ± 0.01 (ppm) respectively. The mean concentrations of cadmium residues were 0.022 ± 0.001, 0.048 ± 0.003 and 0.027 ± 0.003 ppm, respectively. While in case of zinc, the residual levels were 1.97 ± 0.12, 2.369 ± 0.32 and 1.126 ± 0.24 ppm, respectively. Copper residual levels in the examined samples were 0.293 ± 0.08, 0.221 ± 0.03 and 0.08 ± 0.02 ppm, respectively. In case of tin, the residual levels were 1.496 ± 0.30, 1.209 ± 0.26 and 0.379 ± 0.11 ppm respectively.

Key Words: Heavy metals, Canned fish, Egypt

Fish is very important source of protein especially in Egypt where the animal protein is insufficient to meet the requirement of increased population. Nutritionally, fish contains a high biological value and highly digestible proteins, essential amino acids appreciable amounts of cobalt, magnesium, phosphorous, iron and copper.

Now, fish canning industry become well established in Egypt and the locally produced canned fish products are widely distributed in the Egyptian markets. The fish canning process vary with the product being canned, the size and the shape of the container, but generally there are principle steps in common practice and the most important one is the selection and preparation of raw materials for processing to obtain a product which agree with the quality control standards[4].

Industrialization has improved general technology as well as quality of life but has also resulted in increase of the metal concentrations in water[11].

The pollution of the aquatic environment with heavy metals has become a serious health concern during recent years. Untreated municipal and industrial wastes, together with inputs from the atmosphere, are the major sources of heavy metal pollution, especially in areas in close vicinity to industrial and agricultural activities. Industrial and agricultural discharges are

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considered the primary source of heavy metal poisoning of fish[9].

Toxic elements can be very harmful even at low concentration when ingested over along time period. The essential metals can also produce toxic effects when their intake is excessive[3].

For this reason, determination of the chemical quality of aquatic organisms, particularly the contents of trace elements and heavy metals in fish is extremely important for human health. Thus, the aim of this study was to determine the occurrence of lead, cadmium, zinc, copper and tin in some canned fishes (tuna, sardine and mackerel) distributed in Zagazig city markets, Egypt.

A total of 75 random samples of canned fishes (tuna, tardine and mackerel, 25of each) were collected from supermarkets in Zagazig city, Egypt. These samples were examined clinically and inspected at Food Control Department, Faculty of Veterinary Medicine Zagazig University, Zagazig, Egypt.

Standard solutions of lead, cadmium, zinc, copper and tin were purchased from Merck, Darmstadat, Germany. The standards were prepared from the individuals 1000 mg/1 standard in 0.1 N HNO3. Working standards were prepared from the previous stock solutions. One gram from each sample was macerated in screw capped tube. Five millimeters of digestion mixture consists of three parts of nitric acid and two parts of perchloric acid were added to the tissue sample. The tubes were tightly closed and the content was vigorously shacked and allowed to stand overnight at room temperature. The tubes were heated for three hours at water bath adjusted at 70°C to ensure complete digestion of samples. The digestion tubes were vigorously shacked at 30 min interval during heating in water bath. The tube were cooled at room temperature and then diluted with 5 ml deionized water, capped with plastic film and thoroughly mixed. The digested solution was filtered through whatt-man filter paper. The filtrate was collected in Pyrex glass test tubes. These tubes were capped with polyethylene film and kept at room temperature until analyzed for heavy metal contents [8]. The metals were measured using Atomic Absorption Spectrophotometer (AAS) (Perkin-Elmer 2380). Lead (Pb) was measured at 217 nm, while cadmium (Cd) was measured at 228.8 nm, zinc (Zn) was measured at 213.9 nm, copper (Cu) was measured at 324.7 nm and tin (Sn) was measured at 286.3 nm with hollow cathode lamps. The limit of detection was 10 μg/kg for Pb, 1 μg/kg for Cd, 10 μg/kg for Zn, 10 μg/kg for Cu and 1 μg/kg for Sn. The recoveries in different sample materials were recovery rates of 97%, 98%, 96%, 95% and 97% for Pb, Cd, Cu, Sn and Zn respectively. All obtained results were corrected according to the recovery rates. Statistical significances were evaluated using Tukey-Kramer HSD difference test (JMP) (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to be significant.

Water pollution leads to fish contaminated with toxic metals, from many sources, e.g. industrial and domestic waste water, natural runoff and contributory rivers[2,10]. Fish generally accumulate contaminants from aquatic environments, have been largely used in food safety studies. Heavy metals discharged into the marine environment can damage both marine species diversity and ecosystems, due to their toxicity and accumulative behavior[7,12]. Lead reaches the aquatic system because of superficial soil erosion and atmospheric deposition, accumulated in fish. High levels of lead affect gastrointestinal, neuromuscular, renal and hematological systems[1]. Our results declared that the concentration of lead in canned tuna ranged from 0.02 to 0.17 with a mean value of 0.127 ± 0.02 ppm. Concentration of Pb in canned sardine ranged from 0.00–0.03 with a mean level of 0.013 ± 0.004 ppm. Level of Pb in canned mackerel ranged from 0.00–0.05 with a mean value of 0.023 ± 0.01 ppm. Lower Pb concentrations in canned sardine 0.00–5.1 (0.2) μg/kg were reported[5], 0.111 ppm[8], and 10 μg/kg[15]. Higher levels of Pb in canned sardine

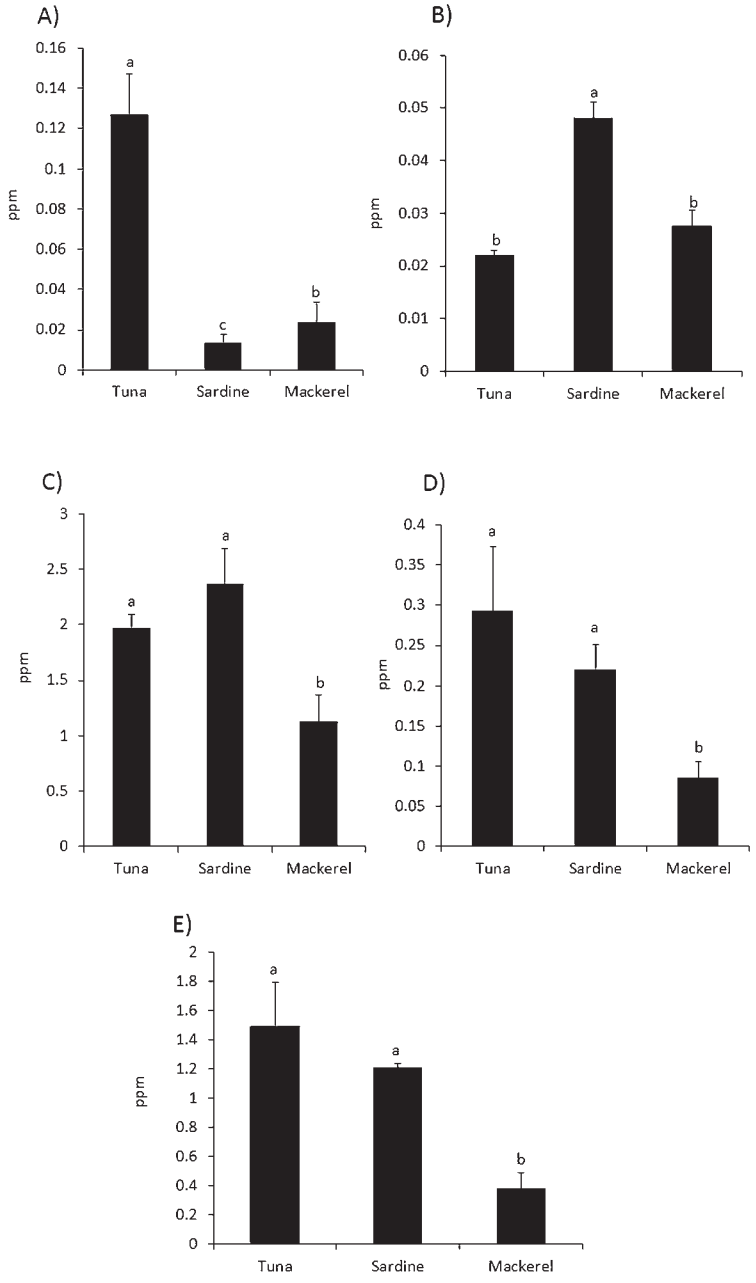

Fig. 1. Heavy metal residues in canned fish distributed in Zagazig city. A) Lead (Pb) B) Cadmium (Cd) C) Zinc (Zn) D) Copper (Cu) E) Tin (Sn) contents (Mean ± SE) in canned tuna, sardine and mackerel (n = 25 each). Heavy metal contents (ppm/wt. weight) were measured using AAS. Pb was measured at 217 nm, while Cd was measured at 228.8 nm, Zn was measured at 213.9 nm, Cu was measured at 324.7 nm and Sn was measured at 286.3 nm. Star mark indicates significant difference ($P < 0.05$).

were recorded 0.77–2.15 μg/g[11] and 2.419 ± 0.282 ppm[13].

Residual levels of Cd in canned tuna ranged from 0.02–0.03 with a mean value of 0.022 ± 0.001 ppm. In examined canned sardine the ranged varied from 0.04–0.06 with a mean level of 0.048 ± 0.003 ppm. Concentration of cadmium in canned mackerel ranged from 0.02–0.04 with a mean level of 0.027 ± 0.003 ppm. Lower results of Cd in canned mackerel as 13 μg/kg were reported[15], higher results were recorded as 0.15 ± 0.01 μg/g[13].

Zinc residual levels in canned tuna ranged from 1.59–2.27 with a mean level of 1.970 ± 0.12 ppm. In canned sardine Zn residues ranged from 1.25–2.99 with a mean level of 2.369 ± 0.32 ppm. In canned mackerel Zn levels ranged from 0.78–2.09 with a mean level of 1.126 ± 0.24 ppm.

The level of Cu in canned tuna ranged from 0.01–0.55 with a mean value of 0.293 ± 0.08 ppm. The minimum, maximum and mean levels of Cu in canned sardine were 0.09, 0.30 and 0.221 ± 0.03 ppm, respectively. Lower levels were recorded 2.07–4.57 μg/g[11], 0.50–1.75 ppm[5]; and 1.9 ± 0.15[14]. The residual concentration of Cu in canned mackerel showed the lowest accumulation level as it was ranged from 0.03–0.15 with a mean level of 0.086 ± 0.02 ppm. Lower levels were recorded by 0.42–1.28 ppm[5], and 1.10 ± 0.10 μg/g[13].

Level of Sn in canned tuna ranged from 0.57–2.41 with a mean level of 1.496 ± 0.30 ppm. Lower result recorded were 0.00 μg/g (not dectable)[6] and 0.04–0.52 mg/kg[5]. In case of sardine, the mean residual level of tin was 1.209 ± 0.26. Mackerel had the lowest residual level of tin, 0.379 ± 0.11 ppm. These differences in the accumulation levels may be attributed to the sample origin, feeding habit and the age of the fish.

References

1- Agency for Toxic Substance and Disease Registry (ATSDR) 2005. Toxicological profile for lead U.S. Department of Health and Humans Services, Public Health Service, Centers for Diseases Control, Atlanta, GA.

2- Arain, M. B., Kazi, T. G., Jamali, M. K., Afridi, H. I. and Jalbani, N. 2007. Factorial designs for As, Cd and Pb ultrasound assisted pseudodigestion of fish muscles followed by electrothermal atomic absorption spectrophotometer, *J. AoAc Interna*., **90** (2): 470–478.

3- Celik, U. and Oehlenschlager, J. 2007. High contents of Cadmium, lead, zinc and copper in popular fishery products sold in Turkish supermarkets. *Food control*, **18**: 258–261.

4- Dural, M., Goksu, M. Z. L. Ozak, A. A. 2007. Investigation of heavy metals levels in economically important fish species captured from the Tuzla lagoon. *Food Chem.,* **102**: 415–421.

5- Ikem, A. and Egiebor N. O. 2005. Assessment of trace elements in canned fishes (mackerel, tuna, salmon, sardines and herrings) marketed in Georgia and Alabama (United States of America). *J. Food Comp. Anal.,* **18**: 771–787.

6- Khansari, F. E., Ghazi-Khansari, M. and Abdollahi, M. 2005. Heavy metals content of canned tuna fish. *Food Chem.,* **93** (2): 293–296.

7- Matta, J., Milad, M., Manger, R. and Tosteson, T. 1999. Heavy metals, lipid peroxidation, and cigateratoxicity in the liver of the caribben barracuda (*Sphyraena barracuda*). *Biol. Trace Elem. Res.,* **70**: 69–79.

8- Premavalli, K. S., Majumder, T. K. and Bawa, A. S. 2007. Quick semi-quantitative test for lead in processed foods. *J. Defense Sci.,* **57** (2): 243–247.

9- Sorensen, E. M. B. 1991. Metal poisoning in fish. CRC press Florida.

10- Tariq, J., Jaffar, M. and Moazzam, M. 1991. Concentration correlations between major cat ions and heavy metals in fish from the Arabian Sea, *Mar. Pollut. Bull.,* **22** (11): 562–565.

11- Tarley, C. R. T., Coltro, W. K. T., Matsushita, M. and de Souza, N. E. 2001. Characteristic levels of some heavy metals from brazilian canned sardines (Sadinella brasiliensis). *J. Food Comp. Anal.,* **14**: 611–617.

12- Turkmen, M., Turkmen, A., Tepe, Y., Tore, Y. and Ates, A. 2009. Determination of metals in fish species from Aegean and Mediterranean Seas. *Food Chem.,* **113**: 233–237.

13- Tuzen, M. 2009. Toxic and essential trace elemental contents in fish species from. *Food chem. Toxicol.,* **47**: 1785–1790.

14- Tuzen, M. and Soylak, M. 2007. Determination of trace metals in canned fish marketed in Turkey. *Food Chem.,* **101**: 1378–1382.

15- Usydus, Z., Szlinder-Richert, J., Polak-Juszczak, L., Kannderska, J., Adamczyk, M., Melesa-Ciecwierz, M. and Ruczynska, W. 2008. Food of marine origin: Between benefits and potential risks. Part I. canned fish on the polish market. *Food Chem.,* **111**: 556–563.

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NOTE

Determination of organochlorine pesticides (OCPs) in the edible offal of Egyptian buffalo

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Abstract

Environmental contamination by OCPs has a great concern, since most of these pesticide compounds are very toxic and harmful to human and ecosystems. This study was conducted to determine the concentrations of OCPs residues in the edible offal (livers, kidneys and tongues) of Egyptian buffalo collected from three locations (Zagazig, Ismailia and Mansoura) in Egypt. Examined samples from Mansura city had the highest OCPs contamination load. Tongues had the highest concentration of these toxic residues in a comparison to livers and kidneys in the examined samples. The overall results showed that OCPs residues did not exceed the Egyptian maximum permissible limits in all of the samples analyzed from the three different locations.

Key Words: Egyptian buffalo, Organochlorine pesticides, Offal

For the past 50 years, awareness has been growing about the threats posed to human health and the global environment by the ever-increasing release of synthesized chemicals especially organochlorine pesticides (OCPs) into the natural environment. Several OCPs are listed on the Stockholm convention on persistent organic pollutants, which is a global treaty to protect human health and the environment from POPs[15)].

Organochlorine pesticides are chemicals used to kill or control pests. They fall into three major classes: insecticides, fungicides, and herbicides (or weed killers). In the 1940s, many chlorinated hydrocarbon insecticides were developed though they did not come into widespread use until the 1950s. Many of the organochlorine pesticides are classified as possible carcinogenic, teratogenic, and neurotoxic; therefore, they are of considerable concern to human, animal and environmental health, causing an array of adverse effects and death[1,3,4,5)]. Organochlorine pesticides (OCPs) have received considerable attention in the last decade since studies have shown extreme persistence of these pollutants in the world-wide environment and accumulation in human and animal tissue. One of the great challenges in food safety is the control of risks associated with

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mixtures of contaminants, which are changing continuously[6].

In Egypt, all types of OCPs have been banned since late 1990, after being used for more than 50 years for agriculture and public health reasons. However due to its cheap price, easy to use, and effectivity on eradication of pests, some kind of OCPs such as DDTs and γ-HCH (lindane) are still used in Egypt, coupled with a lack of law enforcement[10]. Being a large agricultural country, substantial amounts of OCPs are still being illegally used for agriculture and animal production programs in many areas in Egypt. As a result, contamination of food stuffs, especially those having a high fat content such as meat and meat products recorded[8]. Monitoring of OCPs residues in developed countries is a continuous task. However, in Egypt as many developing countries, the available information about OCPs contamination is scarce and if present not regularly updated.

To monitor the impact of such pollution on the public health, we have to establish some biomarkers for this pollution. Buffalo are considered to be good biomarkers for different environmental pollutants, particularly, organochlorine pesticides simply because they have the ability to accumulate some residues of these toxic chemicals in their tissues. Moreover, these animals are domesticated and live under the same environment with human[2]. Thus, the objective of this study was to investigate the current situation of OCPs residues in livers, kidneys and tongues of the Egyptian buffalo collected from three locations (Ismailia, Zagazig and Mansura) in a comparative way.

All experiments using animals were performed according to the guidelines of Zagazig University and Hokkaido University Institutional Animal Care and Use Committee. A total of 135 random samples of livers, kidneys and tongues were collected from male Egyptian buffalo (*Bubalus bubalis*) (3.57 ± 0.48 year old) from 3 locations (Ismailia, Zagazig and Mansura, 45 sample for each location divided as 15 for each of liver, kidney and tongue) in the period of October, 2010 to June, 2011. The samples were collected directly after inspection at Ismailia, Zagazig and Mansoura slaughter houses. The collected samples were purchased from Egypt with permission from the Ministries of Agriculture and animal quarantine departments of both Egypt and Japan. Five grams of each sample were homogenized with anhydrous sodium sulphate and placed into acetone/hexane pre-washed extraction thimble. The samples were extracted in Soxhlet S306AK Automatic Extractor System (Gerhardt, Germany) for 6 h with 150 mL mixture of hexane: acetone (3 : 1 *v*/*v*). Then extracts were concentrated to approximately 2 mL using rotator vacuum evaporator and then diluted to 10 mL with hexane. An aliquot of 20% of the extract was taken for gravimetric lipid determination and the rest was subjected for clean-up process after solvent evaporation. Clean-up was performed on a glass column packed with 6 g of activated florisil (kept overnight at 150°C) topped with anhydrous sodium sulphate. Elution was carried out using 80 mL of hexane containing 30 % (*v*/*v*) dichloromethane. The effluent was concentrated to about 2 mL and then to near dryness under gentle nitrogen flow. The extract was redissolved in 100 μL n-decane and transferred to GC-vials for analysis. Analysis of twenty two OCPs namely HCHs (*α*-, *β*-, *γ*- and *δ*-HCH), HPTs (heptachlor and *cis*- and *trans*-heptachlor epoxide), CHLRs (*cis*- and *trans*-chlordane, oxy-chlordane and *cis*- and *trans*-nonachlor), DDTs (*o*,*p*'- and *p*,*p*'- DDT, DDE and DDD), Drins (aldrin, dieldrin and endrin) and HCB (hexachlorobenzene) were carried out with a gas-chromatography equipped with Ni electron capture detector (GC-ECD: Shimadzu GC-2014, Kyoto, Japan). An EVN-8MS capillary column (30 m length × 0.25 mm I.d, 0.25 μm film thickness; Kanto chemical Co., Japan) was used for separation. 1 μl of each sample was injected in splitless mode. The GC oven temperature was programmed from 100°C held for 1 min., ramped

at 12°C /min to 180°C, then at 4°C/min to 240°C, and finally at 10°C/min to 270°C and held for 5 min. The temperature of injector and detector were 280°C and 320°C, respectively. Helium was used as the carrier gas with a flow rate of 0.1 ml/min and nitrogen as the make-up gas at flow rate of 45 ml/min. All obtained results were corrected according to the recovery rates. Statistical significances were evaluated using Tukey-Kramer HSD difference test (JMP) (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to be significant.

Although most of OCPs are no longer in use, they are still being found as residues and occurring in food now as a result of environmental contamination. Pesticides residues in water, plants and grasses may be ingested by herbivorous animals and eventually find their way into meat[16].

Because of their high thermodynamic stability and lipid solubility, OCPs bind to lipid components in animal tissues, becoming a major route of human exposure when consumed as food, contributing to more than 90% of the daily exposure to these compounds. Animal exposure may arise from direct treatment with pesticides, inhalation of contaminated air, or through ingestion of contaminated forages, herbage and feedstuffs[13]. In bovine tissues after resorption, OCPs enter the liver and are metabolised slowly before they are released into the circulatory system and either finally deposited in fat or excreted. Tissue distribution patterns of OCPs in bovine carcasses varied significantly among seasons, geographic locations and tissues[11]. Screening of OCPs in animal tissues collected from different locations is important because it provides useful information concerning the extent of pollution trends in relation to local origin; it helps to clarify the differences in contamination levels, and to evaluate the health risks associated with the consumption of contaminated food from meat producing animals.

In the current investigation, the concentration of OCPs expressed as ng/g lipid weight (lw) in tissues of buffalo from Ismailia, Zagazig and Mansura is presented in Fig. 1–3. The mean concentrations of ∑HCHs in the examined livers, kidneys and tongues of Ismailia buffalo were 34.97 ± 9.42, 41.97 ± 8.94 and 59.24 ± 24.34 respectively. These values in Zagazig buffalo samples were 79.61 ± 11.69, 70.26 ± 17.42 and 85.52 ± 8.82 respectively. Meanwhile, the residual levels in the samples collected from Mansura were significantly higher as they recorded 89.21 ± 11.57, 247.73 ± 67.12 and 351.57 ± 131.88 in livers, kidneys and tongues respectively (Fig. 1A).

Total Drins in the collected samples from Ismailia city were 18.09 ± 4.83, 27.72 ± 4.85 and 23.85 ± 8.94 in livers, kidneys and tongues respectively. These contents were 14.92 ± 7.57, 11.19 ± 2.66 and 22.92 ± 6.95 in the livers, kidneys and tongues collected from Zagazig. Samples collected from Mansura city showed significantly higher residual levels of ∑Drins, particularly in both kidney and tongue, as the recorded values were 30.27 ± 3.54, 68.13 ± 15.85 and 84.00 ± 25.37 in livers, kidneys and tongues respectively (Fig. 1B).

HCB was detected only samples collected from Ismailia and Mansura. The residual HCB levels in the examined kidneys and tongues from Ismailia were 9.49 ± 3.11 and 23.18 ± 8.82 respectively. In Mansura, HCB was detected only in the examined livers and kidneys with mean concentrations of 10.15 ± 2.67 and 96.47 ± 30.19, respectively (Fig. 1C).

DDTs were only detected in the samples collected from Mansura city; the measured residual levels of ∑DDTs were 18.41 ± 3.58, 35.67 ± 9.97 and 62.83 ± 17.73 in livers, kidneys and tongues respectively (Fig. 2A). However, CHLRs were detected only in liver and kidney samples collected from Ismailia city; ∑CHLRs mean concentrations were 19.08 ± 8.53 and 45.09 ± 6.38 in liver and kidney respectively (Fig. 2B).

Among the detected OCPs in this study, HCHs were the most dominant compounds in the

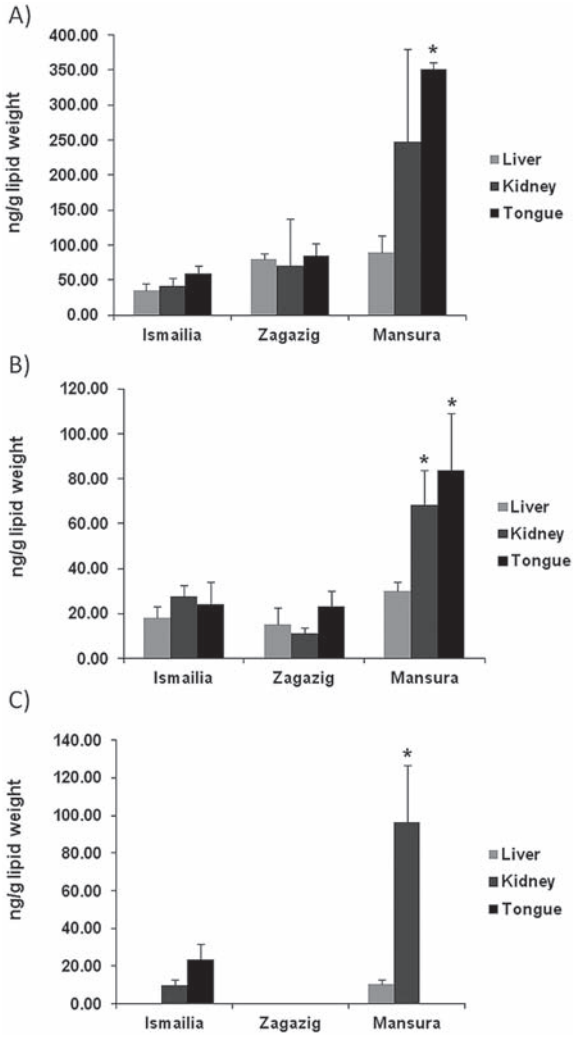

Fig. 1. OCPs contents (ng/g lipid weight) in buffalo edible offal slaughtered in Egypt. A) HCHs contents; B) Drins contents; C) HCB content (ng/g lipid weight) in the liver, kidney and tongue samples (15 each) collected from Ismailia, Zagazig and Mansura slaughter houses. The samples were prepared according to the text. OCPs measurement was carried out in a gas-chromatography equipped with Ni electron capture detector. Data are presented as mean ± SE. Columns carrying asterisk are significantly different than others. $P < 0.05$.

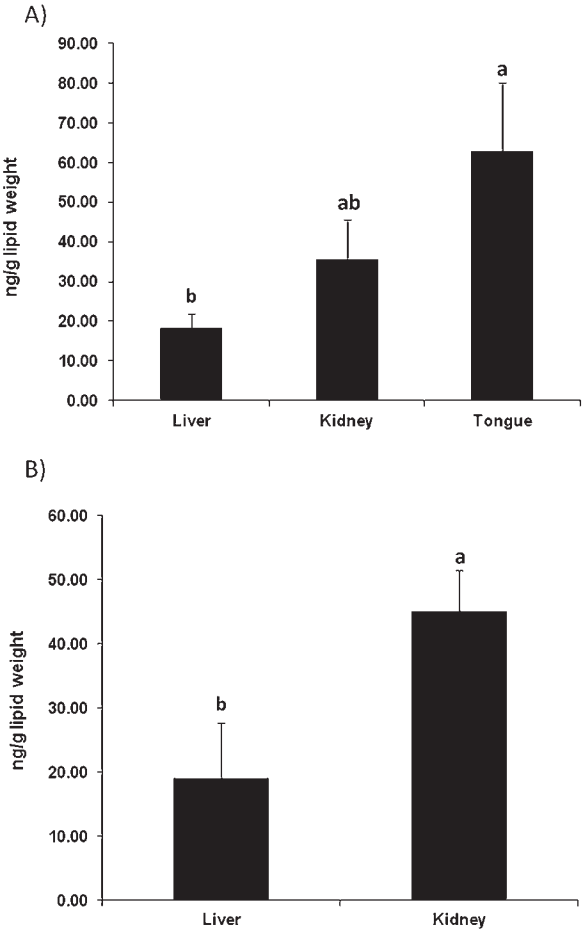

Fig. 2. DDTs and Chlordanes concentration (ng/g lipid weight) in buffalo edible offal slaughtered in Egypt. A) DDTs contents in the liver, kidney and tongue samples (15 each) collected from Mansura slaughter house. B) CHLRs contents (ng/g lipid weight) in the liver and kidney samples (15 each) collected from Ismailia slaughter house. The samples were prepared according to the text. OCPs measurement was carried out in a gas-chromatography equipped with Ni electron capture detector. Data are presented as mean ± SE. Identical letters were not significantly different from each other. $P < 0.05$.

examined tissue samples of buffalo followed by Drins. However, the use of DDTs, HCB and CHLRs is scarce (Fig. 3). Our findings correspond with *Sallam and Morshdy*[12] who reported that HCHs are the most dominant pesticide used in Egypt. Additionally, data reported in this study showed that DDTs are still in use in Mansura city which is mainly agricultural area compared with both Zagazig and Ismailia. The obtained results in this study are comparable with the reported values for OCPs residues in meats from Mexico and Egypt[11,12] however higher values of OCPs were reported in meat from India[9,14].

Interestingly, the tongue samples from Mansura city showed the highest content of HCHs, Drins and DDTs; which may be attributed to the fact that the tongue is considered the first line of defense against xenobiotic exposure.

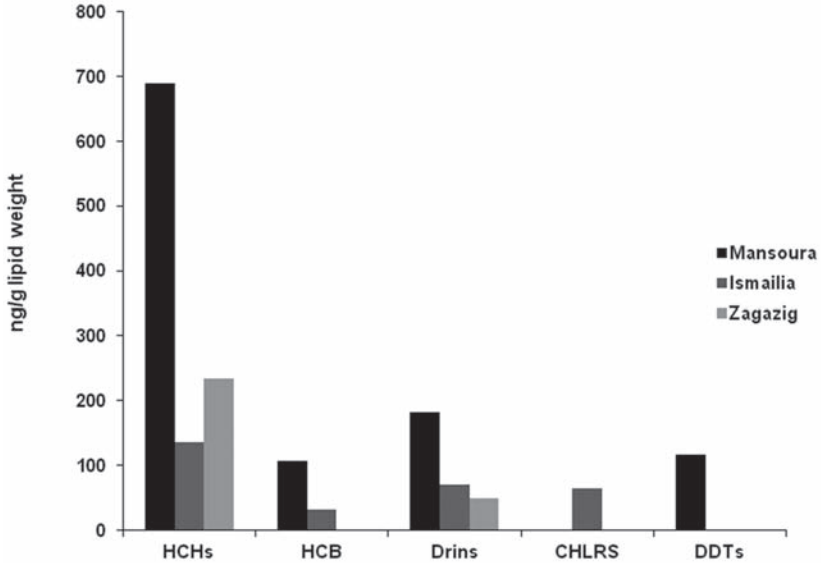

Fig. 3. Concentrations of different OCPs in different locations in Egypt. Pooled data from all analyzed samples

Similar finding was reported in the tongues of camel which showed the highest cytochrome P450 1A1 mRNA expression compared with the kidney and liver[2].

The reported values in this study showed that, the residual concentration of all OCPs detected in buffalo tissues samples were lower than Egyptian recommended maximum permissible limits for HCHs (1 μg/g), Drins (600 ng/g), DDTs (5 μg/g), CHLRs (200 ng/g) and HCB (200 ng/g)[7].

In conclusion, the Overall OCPs residual concentrations detected in all of the contaminated samples analyzed from the three different sampling areas were low and did not exceed the respective maximal permissible limits. However, recent input of HCHs (lindane) and DDTs might still exist in the areas investigated and continuous screening should be carried on.

Acknowlegements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, which was awarded to M. Ishizuka (No. 19671001) and from Ministry Of Higher Education and Scientific Research (MHESR), Egypt which was awarded to Abdallah. F. A. Mahmoud. W. S. Darwish is a recipient for a postdoctoral fellowship from Japanese Society for Promotion of Science (JSPS).

References

1. Albrecht, W. N. 1987. Central nervous system toxicity of some common environmental residues in the mouse. *J. Toxicol. Environ. Health,* **21**: 405–421.
2. Darwish, W. S., Morshdy, A. E., Ikenaka, Y., Ibrahim, Z. S., Fujita, S. and Ishizuka, M. 2010. Expression and sequence of CYP1A1 in camel. *J. Vet. Med. Sci.,* **72**: 221–224.
3. Descampiaux, B., Cotelle, N., Catteau, J. P., Peucelle, C., Leroux J. M. and Erb, F. 1999. Cytotoxicity of lindane and paraquat to human hepatoma cell lines. *Bull. Environ. Contam. Toxicol.,* **62**: 16–24.
4. DHHS. 1998. National Toxicology Program Report on Carcinogens, Eight Edition. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Services, National Institute of Environmental Health Services.
5. Dikshith, T. S., Srivastava, M. K. and Raizada, R. B. 1990. Fetotoxicity of

hexachlorocyclohexane (HCH) in mice: Morphological, biochemical and residue evaluations. *Vet. Hum. Toxicol.,* **32**: 524–527.
6. Dorne, J. L., Fink-Gremmels, J., Alexander, J., Heppner, C., Kass, G. E. and Schlatter, J. 2012. Special issue: Risk assessment of undesirable substances in feed. Toxicol Appl Pharmacol. 2012 Nov 19. pii: S0041-008X(12)00484-X. doi: 10.1016/j.taap.2012.11.011. [Epub ahead of print].
7. EOS (1992b). Maximum limits for pesticide residues in meat. Paper No. 2078. Aldrin and dieldrin. Egyptian Organization for Standardization and Quality Control, Cairo, Egypt.
8. Kannan, K., Tanabe, S., Williams, R. J. and Tatsukawa, R. 1994. Persistent organochlorine residues in foodstuffs from Australia, Papua New Guinea and the Solomon Islands: contamination levels and human dietary exposure. *Sci. Total Environ.,* 15; **153**(1–2): 29–49.
9. Kannan, K., Tanabe, S., Ramesh, A., Subramanian, A. and Tatsukawa, R. 1992. Persistent organochlorine residues in foodstuffs from India and their implications on human dietary exposure. *J. Agric. Food Chem.,* **40**: 518–524.
10. Nasr, N., Arief, M. H., Abdel-Aleem, A. H. and Malhat, F. M. (2009). Persistent Organic Pollutants (POPs) in Egyptian Aquatic Environment. *J. Appl. Sci. Res.,* **5**(11): 1929–1940.
11. Pardío, V., Martínez, D., Flores, A., Romero, D., Suárez, V., López, K. and Uscanga R. 2012. Human health risk of dietary intake of organochlorine pesticide residues in bovine meat and tissues from Veracruz, México. *Food Chem.*, **135**(3): 1873–93.
12. Sallam, K. I. and Morshedy, A. E. M. 2008. Organochlorine pesticide residues in camel, cattle and sheep carcasses slaughtered in Sharkia Province, Egypt. Food Chem., **108**(1): 154–164.
13. Semeena, V. S., Feichter, J. and Lammel, G. 2006. Impact of the regional climate and substance properties on the fate and atmospheric long-range transport of persistent organic pollutants—examples of DDT and gamma- HCH. *Atmos Chem Phys.*, **6**: 1231–1248.
14. Singh, P. P. and Chawla, R. P. 1988. Comparison of sulphuric acid treatment and column chromatographic clean-up procedures for the gas chromatographic determination of organochlorine compounds in some food commodities. J. Chromatogr., **457**: 387–392.
15. Stockholm Convention. Stockholm Convention on persistent organic pollutants (POPs). Geneve: Stockholm convention; 2008 [cited 2009 May 12]. Available from: http://chm.pops.int/Default.aspx.
16. W.H.O, 1990. Public health impact of pesticides used in agriculture. WHO in collaboration with the UNEP. World Health Organization, Geneva.

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NOTE

Forensic Case of Lead Poisoning From a Battery Manufacturing Company in Nakuru, Kenya

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Abstract

Acute sickness involving dairy cattle (n = 5) with a morbidity of 100% occurred in a farm in Nakuru, Kenya. A case study was undertaken with the objective of establishing the cause of the sickness. Samples of blood, soil and industrial waste contained high levels of lead. The symptoms, results of postmortem and history of the case were used to establish the diagnosis of acute lead poisoning. This is a forensic case in court between the owner of the animals and a lead recycling company that dumped the industrial waste that was associated with the poisoning. There could be many unreported cases of lead poisoning in Kenya areas with heavy industrial activities since data on of lead poisoning in Kenya is scanty.

Key Words: Kenya, Lead, Poisoning

Introduction

A dairy farmer reported occurrence of acute sickness involving dairy animals (n = 5) to a veterinarian. The morbidity was 100 % and when the veterinarian visited the farm, he did not come up with a straight forward diagnosis. After consultation and discussion with other veterinarians including a toxicologist, it was decided that a thorough case study was necessary. The objective of the study was to establish the cause of the sudden sickness in order to guide in treatment and management of the disease. Fig. 1 shows the area where the poisoning occurred. Lead is secreted in milk in lactating animals hence it occurrence in dairy cattle is of public health significance.

Materials and Methods

Tentative diagnosis of lead poisoning was made based the initial data base including toxicological history and clinical signs response in acutely poisoned animals. Confirmation of the diagnosis was done by analysis for the toxicant from environmental and biological samples and postmortem examination. Postmortem examination was done at the Veterinary Investigation Laboratories (Nakuru). Blood, industrial waste and soil samples were taken and analyzed for lead level using Atomic

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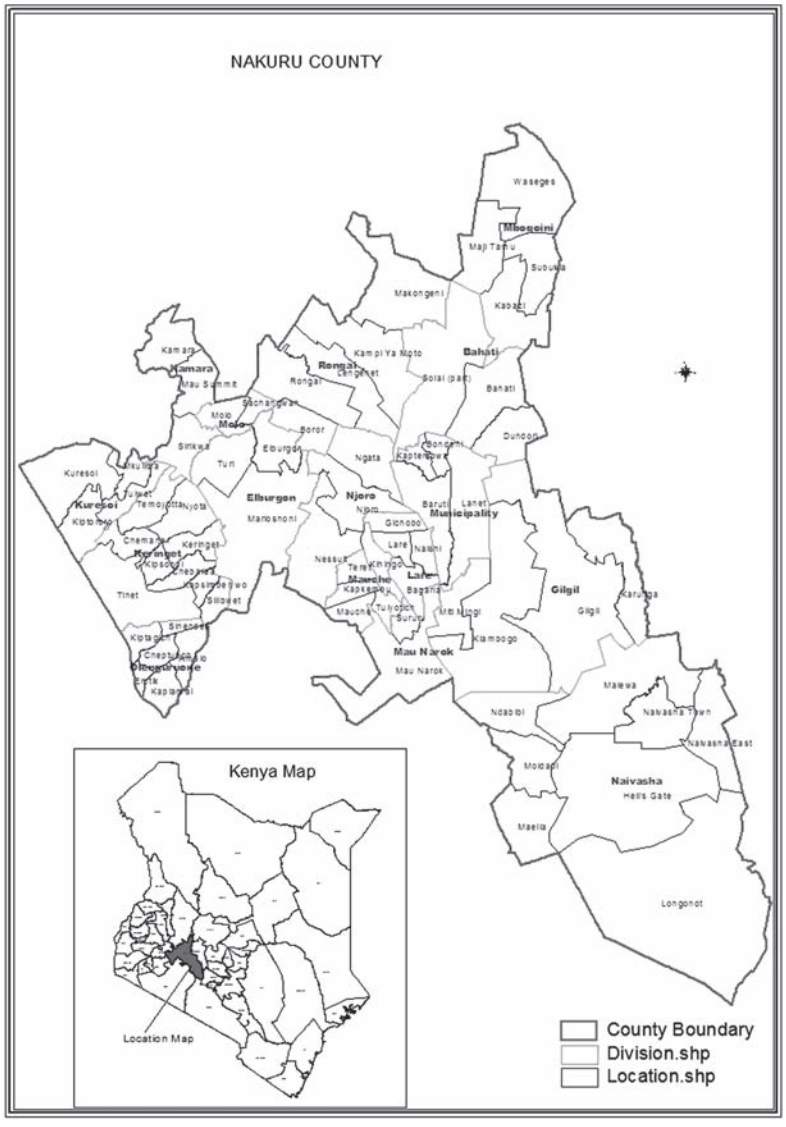

Fig. 1. Map of Kenya showing the location of the study area.

Table 1. Concentrations of lead samples of blood, soil and industrial waste in a case of lead poisoning in Kenya

Sample type	Number (n)	Lead concentration (ppm) Mean (range)
Blood	5	1.47 (0.23–2.30)
Soil	2	42.0 (38.0–46.0)
Industrial waste	2	64,670 (61,236–68,104)

Absorption Spectrophotometric (AAS) technique. This analysis was done at the University of Nairobi, Department of Public Health, Pharmacology and Toxicology and Ministry of Environment and Mineral Resources laboratories (Kenya).

Results

A tentative diagnosis of lead poisoning was made based on clinical signs of salivation, staggering gait, bellowing, aggressive behavior, rolling back of eyeballs and colic. This was supported by history that a driver of a lorry from a lead recycling company was witnessed damping industrial waste and the affected animals were seen licking the industrial waste and feeding on the pasture at the damping site. Samples obtained from the animals and the environment was found to contain toxic levels of lead (Table 1). The animals did not respond to treatment with repeated doses of calcium EDTA and broad spectrum antibiotics. At postmortem, gastrointestinal tracts were inflamed and corroded. When the owner of the animals was informed of the diagnosis, she decided to sue the battery manufacturing company for loss of the animals. The dairy unit was the only source of livelihood for the affect farmer. The case attracted the attention of environmentalists, The National Environmental Management Agency NEMA) and The Public Complained Committee to made visits to the Xiang Hui lead recycling factory. The case was widely reported in press and print media in Kenya.

Discusion, Conclusions and Recommendations

There has been an increased occurrence of environmentally related cases of poisoning in livestock, fish, wildlife and human in Kenya. The most commonly identified poisons or suspected poisons have been pesticides, heavy metals and toxic plants. In many countries, lead is among the most widely encountered toxicant. Small amount of lead kill cattle. Cattle can drink used oil, lick grease or get exposed to other sources of lead in the environment. In the stomachs of cattle, lead is converted into poisonous salts. In lactating animals, lead is secreted into milk. Lead combines with red blood cells and bone marrow. It damages the small blood vessels, causing bleeding, and deprives the nerves, the brain and other organs of oxygen. Although clinical signs of poisoning normally precede death, most animals are simply found down or dead on the pasture. Muscle tremors, excitement, mania, blindness or convulsions may also be seen. After the onset of signs, cattle with acute poisoning usually die within 12 to 24 hours.

The case report in the current study is a forensic case that is in court. Probably there are many unreported cases of lead poisoning in Kenya especially in areas with heavy industrial activities like Nakuru. Information on the epidemiological aspects of lead poisoning in Kenya is scanty. It is recommended that studies are required to establish the status of lead pollution in Kenya and assess the risk of lead exposure to human through consumption of contaminated animal products such as milk and honey.

References

1. Lanphear, BP; Hornung, R; Khoury, J; Yolton, K; Baghurst, P; Bellinger, DC; Canfield, RL; Dietrich, KN *et al*. (2005). "Low-Level Environmental Lead Exposure and Children's Intellectual Function: An International Pooled Analysis". *Environmental health perspectives* **113** (7): 894–9.

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NOTE

Effects Of Endosulfan Pesticide On Toad

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Abstract

The lethal and sublethal toxicity of Endosulfan on the African toad, *Bufo regularis* were evaluated to assess changes in behaviour and energy reserves. 96 hours LC_{50} was 0.730 mg/l while the estimated safe concentration was 0.07 mg/L indicating the high toxicity of the insecticide. Toads exposed to lethal concentrations of endosulfan showed dose-dependent behavioural abnormalities with more pronounced poisoning symptoms occurring at higher concentrations. The pesticide caused differential increase in serum glucose levels with a concomitant reduction in liver glycogen indicating disorders in carbohydrate metabolism due to pesticide induced stress and hence can serve as suitable biomarkers in pesticide toxicity studies.

Key Words: Amphibian, Endosulfan, Toxicity

In agriculture, endosulfan although banned in some countries is widely used for the control of insect pests[3]. However, because it is non-specific, it can negatively impact non-target organisms like amphibians[3]. The aim of this study was to investige the the toxicity of Endosulfan, to the African toad, *Bufo regularis*. mortality, changes in behaviour and energy reserves were investigated. Ecological concentrations, 0.25, 0.50, 0.75 and 1 mg/L, for the lethal test and 0.01, 0.02, 0.03 and 0.04 μg/l, for the sublethal test were prepared. Adult amphibians were exposed for 96 h for the lethal tests and for 28 days for the sublethal tests. Observations were made for mortality and behavioural changes for the lethal test while changes in serum glucose and liver glycogen were assessed for the sublethal test. Serum glucose level was estimated by glucose oxidase method while liver glycogen was determined by Anthrone method[1].

No mortality was observed in the control for the 96-h acute toxicity test. Mortality however occurred in toads exposed to varying endosulfan concentrations (0.25, 0.50, 0.75, 1.0 mg/L) with highest mortality at the highest concentrations, suggesting concentration graded lethality. The 96 hours LC_{50} was 0.730 mg/l while the estimated safe concentraton was 0.07 mg/L. Endosulfan, using toxicity ratings[2] could thus be classified as highly toxic to amphibians. High mortality recorded within the short-term of exposure may

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be due to increased energy demand of detoxication processes, as described by Wiegand *et al.*[4] Behavioural changes observed were characterized with initial hyperactivity, loss of coordination in both front and hind limbs, erratic swimming, unusual retention of water, prolonged and motionless laying down on the aquarium bottom. The behavioural anomalies exhibited by the toads may be an attempt to be relieved from such stressful environment[4]. Toads exposed to Endosulfan showed dose dependent elevations in serum glucose levels and a concomitant reduction in liver glycogen levels. The significant increase in glucose level and reduction in liver glycogen may have resulted from glyconeogensis to provide energy for the increased metabolic demands imposed by endosulfan stress.

References

1. Ezemonye, L. I. N. and Ilechie, I. 2007. Acute and chronic effects of organophosphate pesticides (Basudin) to amphibian tadpoles (*Ptychadenabibroni*). *African Journal of Biotechnology*. 6(11): 1554–1568.
2. Morgan, R. E., Brunson, M. W., 2002. Toxicities of agricultural pesticides to selected aquatic organisms. Southern Regional Aquaculture Center. http://agpublications.tamu.edu/pubs/efish/4600fs.pdf
3. US Environmental Protection Agency (US EPA) 2000.Office of Prevention, Pesticides and Toxic Substances. http://www.epa.gov/pesticides/reregistration/endosulfan/endocrine_response.
4. Wiegand C., Pflugmacher S., Oberemm A., Meems N., Beattie K., Steinberg C. E. W. and Codd, G. A. 1999. Uptake and effects of microcystin on detoxication enzymes of early life stages of the zebrafish *(Daniorerio)*. *Environ. Toxicol.* 14: 89–95.

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NOTE

Distribution of metals in organs of *Clarias gariepinus*, *Heterobranchus bidorsalis*, and *Chrysichthys nigrodigitatus* from the Offin River at Dunkwa-on-Offin, Ghana

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Abstract

All heavy metals are potentially harmful to most organisms at some level of exposure and absorption. Concentrations of Co, Cr, Cu, Ni, Zn, Hg, Cd, As, and Pb were determined by atomic absorption spectrophotometry (AAS) in three fish species (*C. gariepinus*, *C. nigrodigitatus*, and *H. bidorsalis*) from the Offin River in Dunkwa township, Ghana. In the fish species, gills, livers, and muscles were analyzed. The metal that recorded the highest concentration was Zn, which was highly accumulated in the liver of *C. gariepinus*, but had the lowest concentration in the muscles of *C. nigrodigitatus*.

Key Words: atomic absorption spectrophotometry, catfish, metals

Heavy metals have a particular significance in ecotoxicology since they are highly persistent, and all have the potential to be toxic to living organisms[12]. Exposure of animals to either high levels of toxic metals (such as Cd and Pb) or less than optimal levels of essential microelements (such as Cu, Co, and Zn) can cause adverse effects such as reproductive impairment, physiological abnormalities, behavioral modifications, or even death[11]. The region of accumulation of heavy metals within fish varies with route of uptake, type of heavy metals, and the species of fish concerned[1]. Carnivores at the top of the food chain, including humans, obtain most of their heavy metal burden from the aquatic ecosystem by consuming contaminated fish[2].

Fish from the Offin River at Dunkwa township in Ghana supplement the protein requirements of the inhabitants within the area; thus, the contamination of these animals in the aquatic environment by heavy metals is viewed with serious concern. Unfortunately, alluvial gold mining is performed along the river and this could pollute the fish in the Offin River. *Clarias gariepinus*, *Heterobranchus bidorsalis*, and *Chrysichthys nigrodigitatus* were selected for

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this study because they constitute a large population in the Offin River, and, therefore, their metal concentrations are of interest from the human perspective. The objectives of this study were to examine the accumulated levels of heavy metals in muscles, gills, and livers of *Clarias gariepinus*, *Heterobranchus bidorsalis*, and *Chrysichthys nigrodigitatus* from the Offin River at Dunkwa-on-Offin as well as compare the metal concentrations among the three species of fish.

Samples were collected between the months of May and August 2010. Samples of three fish species, *Clarias gariepinus*, *Heterobranchus bidorsalis*, and *Chrysichthys nigrodigitatus*, were obtained from commercial catches from the River Offin at Dunkwa. The samples were kept in an ice box and transported to the Kwame Nkrumah University of Science and Technology (KNUST) chemistry laboratories on the same day. The gill, liver, and muscle of the fish samples were stored in refrigeration at −20°C. Fish samples were transported to the Laboratory of Toxicology, School of Veterinary Medicine, Hokkaido University, Japan, for chemical analyses.

Metals were extracted from the gills, liver, and muscle of fish samples by acid digestion using the method of Nakayama *et al*[10]. A reagent blank was prepared by following the same procedure. The metal concentrations were also determined with an Atomic Absorption Spectrometer (AAS, Z-2010) using the method of Nakayama *et al*[10]. For the measurement of Cr, Co, Cu, Ni, Zn, Pb, Cd, and As, the AAS (Z-2010) was used. Total Hg was determined in all the samples by using the Mercury/MA-3000 Mercury analyzer (Nippon Instrument Corporation, NIC, Tokyo, Japan) after calibration. Measurements of Hg were performed on raw samples without any pre-treatment.

The coefficient of conditions (K) in fish was calculated for each fish sample using the formula: $K = W \times \frac{10^5}{L^3}$, where, K = coefficient of condition, W = weight in grams, and L = body length in millimeters[3].

Statistical analyses were performed using JMP 9 (SAS Institute, Carry, NC, USA). Data were normalized by base 10 logarithm transformations. Steel-Dwass tests were used to analyze differences among the concentrations of metals in fish species. Principal Component Analysis (PCA) was used to identify potential relationships among species and contamination levels as well as the weight and length of the fish species.

There were no significant differences among Hg concentration in the livers of *C. gariepinus*, *H. bidorsalis*, and *C. nigrodigitatus* (Steel-Dwass test, $p < 0.05$). Also, a similar observation occurred in the muscle and gill samples of the three fish species. The coefficient of conditions (K) in *C. nigrodigitatus*, *H. bidorsalis*, and *C. gariepinus* showed no significant difference among the three species of fish.

Mercury concentrations in the liver and muscles of all the three fish species were higher than the threshold value of 0.5 μg/g set by the Egyptian Organization for standardization and Quality Control. The mean concentrations of Cu in the livers of *C. nigrodigitatus*, *H. bidorsalis*, and *C. gariepinus* were greater than the FAO permissible limit. The mean concentrations of Zn in the livers of *C. nigrodigitatus*, *H. bidorsalis*, and *C. gariepinus* were higher than the FAO permissible limit. Arsenic concentration in the livers of *C. nigrodigitatus* could be considered harmful to the fish since the dry weight exceeded 0.5 μg/g. Regardless of the fish from which the samples were collected, the level of Zn was the highest among all the metals in each organ.

Acknowledgments

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References

1. Begum, A., HariKrishna, S., Khan, I. 2009. Analysis of Heavy metals in Water, Sediments and Fish samples of Madivala Lakes of Bangalore, Karnataka. Int. J. of Chem. TechResearch. 1(2): 245–249.
2. Canli, M., AY, Ö. Kalay M. 1998. Levels of Heavy Metals (Cd, Pb, Cu, Cr and Ni) in Tissue of Cyprinus carpio, Barbus capito and Chondrostoma regium from the Seyhan River, Turkey. Tr. J. of Zoo. 22: 149–157.
3. Choongo, C. K., Syakalima, S. M., Mwase, M. 2005. Coefficient of condition in relation to copper levels in muscle of serranochromis fish and sediment from the Kafue river, Zambia. Bull. Environ. Contam. Toxicol. 75: 645–651.
4. Egyptian Organization for standardization and Quality Control. 1993. Maximum levels for heavy metal Contamination in food. No. 2360. Cairo, Egypt: EOSQC.
5. Eisher, R. 1993. Zinc hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish Wildlife Service. 85 (1.10): 90.
6. FAO, (Food and Agriculture Organization), 1983. Compilation of legal limits for hazardous substances in fish and fishery products, FAO fishery circular 464, 5–100.
7. Federal Standards. 1989. Nature Protection Soils Classification of Chemical Species for Detection of Pollution, Federal Standards 174102–83. Moscow (Russian).
8. McCluggage, D. 1991. Heavy Metal Poisoning, NCS Magazine, Published by The Bird Hospital, CO, U.S.A. (www.cockatiels.org/articles/Diseases/metals.html).
9. Muramoto, S. 1981. Variations of some elements in cadmium-induced malformed fish, Bull. Environ, Contam. Toxicol. 27: 193–200.
10. Nakayama, S. M. M., Ikenaka, Y., Muzandu, K., Choongo, K., Yabe, J., Muroya, T., Ijiri. S., Minagawa, M., Umemura, T., Mayumi Ishizuka, M. 2012. Geographic Information System-Based Source Estimation of Copper Pollution in Lake Itezhi-tezhi and Metal-Accumulation Profiles in Oreochromis spp. from Both Field and Laboratory Studies. Arch Environ Contam. Toxicol. DOI 10.1007/s00244–012–9802–3.
11. Nriagu, J., Boughanen, M., Linder A., Howe, A., Grant, C., Rattray, R., Vutchkov, M. and Lalor., G. 2009. Levels of As, Cd, Pb, Cu, Se and Zn in bovine kidney and livers in Jamaica. Ecotoxicol. and Environ. safety 72: 564–571.
12. Öztürk, M., Özözen, G., Minareci, O., Minareci, E. 2009. Determination of heavy metals in fish, water and sediments of Avsar Dam Lake in turkey. Iran. J. Environ. Health. Sci. Eng., 6 (2), 73–80.
13. Sivapermal, P., Sankar, J.V., Nair Viswanathan, P.G. 2007. Heavy metal concentrations in fish, shellfish and fish products from internal markets of India visà-vis international standards. Food Chem., 102: 612–620.
14. USDA, 2006. Foreign Agricultural Service GAIN Report Global Agriculture Information Network Voluntary Report-public distribution GAIN Report Number CH 6064, China Peoples Republic of FAIRS Product.
15. USFDA, 1993. Food and drug administration, Guidance document for nickel in shellfish. DHHS/PHS/ FDA/CFSAN/office of seafood, Washington D.C.
16. Ikem, A., Egiebor, N. O., Nyavor, K. 2003. Trace elements in water, fish and sediment from Tuskegee Lake, Southeastern USA. Water, Air, and Soil Pollution 149: 51–75.
17. Vinas, P., Pardo-Martinez, M., Lopez-Garcia, J., Hernandez-Cordoba, M. 2002. Rapid determination of mercury in food colorants using electro thermal atomic absorption spectrometry with slurry sample introduction. J. Agric Food Chem. 50: 949–954.

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NOTE

Determination of benzo[a]pyrene levels in ambient air and the source of polycyclic aromatic hydrocarbons using a diagnostic ratio method in Ghana

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants produced from incomplete combustion of fuel or vegetation fires. Their presence in air deserves attention because they can produce carcinogenic and mutagenic effects. As an industrialized and economically significant city in Ghana, Kumasi has been subject to heavy anthropogenic influences due to rapid economic development and urbanization leading to a greater fuel combustion rate. Airborne particulate samples were collected on filters using a Sibata air sampler and analyzed by gas chromatography-mass spectrometry (GC-MS). Our results indicated that air from the city center can be classified as highly polluted with benzo[a] pyrene (B[a]P). The diagnostic ratios of the results showed that PAHs in the air samples were mainly from fuel combustion.

Key Words: air, diagnostic ratios, polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) refer to a group of compounds comprised of varying numbers of carbon and hydrogen atoms connected in ring-like forms. They consist of two or more fused benzene rings in linear, angular, or cluster arrangements. Most PAHs entering the environment are formed unintentionally during burning of fossil fuel, biomass, wood, and brushwood[8].

Exposure to PAHs occur mainly by inhalation of contaminated air and ingestion of soil, food, and contaminated water[1,6]. Although food can be contaminated by environmental PAHs (i.e., PAHs from air, dust, and soil), PAHs in food are mainly formed during processing and food preparation, for example, during smoking, roasting, baking, drying, frying, or grilling[9]. Foods grown in areas with PAH-contaminated soil or air may contain higher levels of PAHs. Due to their ubiquitous occurrence, recalcitrance, and suspected

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carcinogenicity and mutagenicity, PAHs are included in the US Environmental Protection Agency (EPA), Environmental Monitoring Assessment, and the European Union priority lists of pollutants. The US EPA has fixed 16 parent PAHs as priority pollutants[2].

The objectives of this study were to determine the levels of benzo[a]pyrene (B[a]P) in air samples and to establish the possible sources of PAHs in air samples using a diagnostic ratio method.

The air samples collected were extracted for PAHs with a 1 : 2 v/v acetone-hexane mixture using a soxhlet extractor. The extracts were dehydrated by filtering through anhydrous sodium sulphate. PAHs analyses were performed using an AS3000 Gas Chromatograph (GC) coupled with a Thermo Scientific Mass Selective Detector operating in the electron impact mode (GC-MS). The selective ion monitoring mode was used for quantification. A GC capillary column (ENV-8MS, 30 m, 0.25 mm inner diameter, 0.25 μm film thickness) was used for separation. Helium gas was used as the carrier gas at a constant flow rate of 1.2 ml/min. Eight percent polycarborane-siloxane was used as the stationary phase. Injection temperature for the GC-MS analysis was 280°C. Temperature programming for the column was as follows: the initial temperature of 90°C was held for 1 min, increased to 280°C at a ramp rate of 10°C/min, and finally, the temperature was further increased to 320°C at a ramp rate of 5°C/min and held for 10 min.

The mean concentration of B[a]P from the Kwame Nkrumah University of Science and Technology (KNUST) Campus, Kumasi-Ghana was compared to similar work done from the Tunghai University Campus (THUC) in Taiwan, which recorded mean B[a]P concentrations of 3.0 $\pm$ 5.9 ng/m^3 [7]. The results from THUC were found to be 150 times higher than that from the KNUST Campus. Since no corresponding standard was available for Ghana, this study adopted the British and Swedish standards as the basis for determining the quality of B[a]P in the air samples from the KNUST Campus. The recommended B[a]P value is 0.25 ng/m^3 under United Kingdom Air Quality Standards (EPAQS)[5], while the Swedish guideline value for B[a]P in the air is 0.1 ng/m^3 [3]. The mean B[a]P concentration in the air samples from the KNUST Campus was less than the standard set by the United Kingdom and five times less than the Swedish standard for B[a]P in the air. The individual concentrations of B[a]P in the air samples from the KNUST Campus were all below the Swedish and British standards. Based on this criterion, air from the KNUST Campus was of good quality and inhabitants of the KNUST Campus could be said to be safe from the deleterious effects of B[a]P, since the concentrations in the air samples were all below the British and Swedish standards.

The mean concentration of B[a]P in air samples from Taichung Industrial Park (TIP), Taiwan was 9.0 $\pm$ 25.4 ng/m^3 [7]. This was higher than that from the city center. On the other hand, the mean concentrations of B[a]P in air samples from the city center were 14.69 and 37.4 times higher than the air quality standard for B[a]P concentrations in the United Kingdom (0.25 ng/m^3) and Sweden (0.1 ng/m^3), respectively. The mean concentration of B[a]P from the city center was markedly higher than the mean concentration of B[a]P from the KNUST campus; therefore, the air from the city center can be classified as highly polluted with B[a]P.

Two pairs of PAH ratios, fluoranthene/(fluoranthene + pyrene), herein denoted as flu/(flu+pyr), and indeno(1,2,3,-c,d)pyrene/(indeno(1,2,3,-c,d)pyrene + benzo(g,h,i)perylene), herein denoted as IDP/(IDP + BghiP), are commonly used to distinguish between combustion sources. The flu/(flu + pyr) ratios between 0.40 and 0.50 are defined as fuel combustion sources, whilst IDP/(IDP + BghiP) ratios greater than 0.5 are indicative of wood, grass, or coal combustion sources[4]. Samples were characterized by IDP/(IDP + BghiP) ratios, and a higher percentage were between 0.2 and 0.5, which indicated a pyrogenic origin of PAHs derived from fuel

combustion. This was confirmed by a flu/(flu + pyr) ratio between 0.4 and 0.5, implying the same fuel combustion source. Some samples exhibited ratios greater than 0.5 for IDP/(IDP + BghiP), which were more typical of wood or grass combustion. The diagnostic ratio charts showed that PAHs in the air samples from the city center were mainly from fuel combustion.

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References

1) Barranco, A., Alonso-Salces, R. M., Crespo, I., Burreta, L. A., Gallo, B., Vicente, F., and Sarobe, M. 2004. Polycyclic aromatic hydrocarbon content in commercial Spanish fatty foods. *J. Food Prot*., **67**: 2786.
2) Baumard, P., Budzinski, H., and Garrigues, P. 1997. Analytical procedure for the analysis of PAHs in biological tissues by gas chromatography coupled to mass spectrometry: Application to mussels. Fresenius *J. Anal. Chem*., **359**: 502–509.
3) Bostrom, C. E., Gerde, P., Hanberg, A., Jernstrom, B., Johanson, C., Kyrklund, T., *et al*. 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. *Environ. Health Perspect.*, **110**(supll 3): 451–89.
4) Bucheli, T. D., Blum F., Desaules, A., and Gustafsson, O. 2004. Polycyclic Aromatic Hydrocarbons, black carbon and molecular markers in soils of Switzerland. *Chemosphere*, **56**: 1061–1076.
5) Dimashki, M., Lim, L., Harrison, R., and Harrad, S. 2001. Temporal trends, temperature dependence, and relative reactivity of atmospheric polycyclic aromatic hydrocarbons. *Environ. Sci. Tech*., **35**: 2264–7.
6) Dissanayake A. and Galloway T. S. 2004. Evaluation of fixed wavelength fluorescence and synchronous fluorescence spectrophotometry as a biomonitoring tool of environmental contamination *Mar. Environ. Res*., **58**: 281.
7) Guor-Cheng F., Yuh-Shen W., Pi-Cheng F. P., I-Lin Y., and Ming-Hsiang C. 2004. Polycyclic aromatic hydrocarbons in the ambient air of suburban and industrial regions of central Taiwan. *Chemosphere*, **54**: 443–452.
8) Manoli, E., Samara, C., Konstantinou, I., and Albanis, T. 2000. Polycyclic aromatic hydrocarbons in the bulk precipitation and surface waters of Northern Greece. *Chemosphere*, **41**: 1845–1855.
9) Phillips D. H. 1999. Polycyclic aromatic hydrocarbons in the diet, Mutat. Res. Genet *Toxicol. Environ. Mut.*, 443: 139–47.
10) Smith, I. M. 1984. PAH from Coal Utilization-Emissions and Effects. Report No. ICTIS/TR29; London, *IEA Coal Res*., pp. **18**

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NOTE

Heavy metal pollution in Japanese seabirds

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Abstract

It is reported that seabirds accumulate high levels of metals, prompting concerns regarding poisoning. The present study investigated the accumulation patterns of metals in tissues among four species of seabirds (*Fratercula corniculata, Uria lomvia, Puffinus tenuirostris*, and *Fulmarus glacialis*). Furthermore, we focused on Slaty-backed Gulls, which accumulated high levels of cadmium and mercury, and compared the areal differences.

Geographic variation of metal levels could also contribute to differences in metal accumulation levels in these bird species. Therefore, the concentrations of metals in seabirds are considered to reflect their habitat.

There are differences in the accumulation pattern among the seabird species. The high accumulation of metals could affect seabirds even if they do not show any symptoms.

Key Words: heavy metals, seabird

It has been reported that marine animals accumulate high concentrations of environmental chemicals[2]. In recent years, the numbers of marine mammals have been decreasing and one of the factors that has contributed to such a decline is metal pollution from mercury, lead, and cadmium. Seabirds also accumulate high levels of these chemicals, prompting concern regarding poisoning. Based on the current situation, we investigated the accumulation patterns of metals in tissues among four avian species of seabirds (*Fratercula corniculata, Uria lomvia, Puffinus tenuirostris,* and *Fulmarus glacialis*) by comparing the accumulated metal concentrations with the stable isotope. Furthermore, we focused on Slaty-backed Gulls (*L. schistisagus*), which accumulated high levels of cadmium and mercury in the tissues, and compared the areal differences. We also examined the relationship between metal

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concentrations in the tissues and their stomach contents and dietary metal levels.

We collected the livers and kidneys of Slaty-backed Gulls (*L. schistisagus*) (n = 20) at Teuri Island, Hokkaido, which were culled for the purpose of conservation of Common Murre (*Uria aalge*) breeding. We also collected the livers and kidneys of Horned Puffin (*F. corniculata*) (n = 2), Brünnich's Guillemot (*U. lomvia*) (n = 3), Short-tailed Shearwater (*P. tenuirostris*) (n = 11), and Northern Fulmar (*F. glacialis*) (n = 2), which were by-caught by fishermen's nets in the Bering Sea. We also focused on Slaty-backed Gulls at Teuri Island. Teuri Island is known as an important place where rare birds breed and live. To understand the source of accumulated toxic metals such as Hg and Cd, we analyzed concentrations of metals in the stomach contents and dietary food, including the Japanese Anchovy (*Engraulis japonica*) and Sea Urchin (*Anthocidaris crassispina*) that live around Teuri Island.

For metal analysis, samples were dried for 15 h at 50°C and digested with HNO_3 and H_2O_2 in a microwave digestion system (Speedwave two, Berghof, Germany). The concentrations of heavy metals (Hg, Cd, Cr, Ni, Pb, Cu, Zn, Co, and As) were measured using a Mercury Analyzer-3000 (MA-3000, Nippon Instrument Corporation, Tokyo, Japan) for total mercury and an Atomic Absorption Spectrophotometer (AAS) (Z-2010, Hitachi High-Technologies Corporation, Tokyo, Japan) for the other metals.

Higher concentrations of Hg, Cd, and As were detected in the liver of Slaty-backed Gulls (Hg: 4.93 mg/kg dry wt for median, 12.88–1.94 mg/kg dry wt for range; Cd: 4.14 mg/kg dry wt for median, 8.84–1.33 mg/kg dry wt for range; As: 0.17 mg/kg dry wt for median, 0.52–0.03 mg/kg dry wt for range) and Northern Fulmars (Hg: 23.1 mg/kg dry wt for median, 39.7–6.6 mg/kg dry wt for range; Cd: 20.19 mg/kg dry wt for median, 26.74–13.64 mg/kg dry wt for range; As: 0.21 mg/kg dry wt for median, 0.24–0.18 mg/kg dry wt for range) compared to other bird species. Food habitat was considered key to the determination of the accumulation pattern. Based on the analysis of stomach contents and diet, including the Japanese Anchovy and Sea Urchin, we also detected high accumulation levels of these metals.

Geographic variation of metal levels could also contribute to differences in metal accumulation levels in these bird species. Therefore, the concentrations of such metals in birds are considered to reflect their habitat.

Although our study showed the lower level compared with the concentration which has the risk of ill, such as the disorder of the nervous, circulatory, and endocrine systems from Hg poisoning[3] and renal lesions from Cd poisoning[1], it does not indicate that there are no risks from such metal accumulation. In fact, the concentrations of Hg and Cd in Slaty-backed Gulls from Hokkaido were higher than other species from the same Hokkaido area.

The present study showed that there are large species and regional differences in accumulation levels of metals and metalloids, especially Hg, Cd, and As, among the investigated seabird species. There is a possibility that highly accumulated metals and metalloids may cause toxicological effects on seabirds, but few studies have been reported to support this. Further research from toxicological perspectives is needed.

References

1) Elliott, J. E., Scheuhammer, A. M., Leighton, F. A., and Pearce, P. A. Heavy Metal and Metallothionein Concentrations in Atlantic Canadian Seabirds. Arch Environ Contam Toxicol. 1992 Jan; 22(1): 63–73.
2) Muir, D., Braune, B., DeMarch, B., Norstrom, R., Wagemann, R., Lockhart, L., Hargrave, B., Bright, D., Addison, R., Payne, J., and Reimer, K. Spatial and temporal trends and effects of contaminants in the Canadian Arctic marine ecosystem: a review. Sci Total Environ. 1999 Jun 1; 230(1–3): 83–144. Review.
3) UNEP (2002) Global mercury assessment. United Nations Environmental Programme—Chemicals, Geneva, Switzerland.

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NOTE

Metabolism of pyrene, a polycyclic aromatic hydrocarbon in freshwater turtles

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Abstract

Reptile population decrease is an alarming trend all around the world. Yet little is known about the role of xenobiotics in this decrease. In this study, we investigated the metabolism of pyrene in three freshwater turtle species (Red-EARED sliders (*Trachemys scripta elegans*), Chinese pond turtles (*Mauremys reevesii*) and Chinese softshell turtles (*Pelodiscus sinensis*). Compared to other vertebrates, all turtles showed an unique metabolite distribution, pyrene-1-sulfate being the main metabolite. The observed low phase II enzyme metabolic rates raises the question of the effect of long-time exposure.

Key Words: Metabolism, Pyrene, Turtle

Reptiles are one of the most endangered and, at the same time, understudied vertebrate groups. Wild populations are threatened by hunting, decrease of habitats, and, among other things, environmental chemicals[1,2]. Yet, with the exception of endocrine distruptors, we know little about the effects and mechanisms of xenobiotics on reptiles.

In this study, we analyzed the metabolites of pyrene, a characteristic member of the polycyclic aromatic hydrocarbons (PAHs) in three freshwater turtle species, as isolated freshwater reptile populations are extremely vulnerable to local catastrophes. Compared to other vertebrate groups, phase II enzyme activities have not been characterized in turtles, and we lack knowledge about the mechanism of PAH metabolism.

PAHs are one of the most abundant organic pollutants in our environment. The toxicity of these molecules varies between species, exposure routes, and the age of the target animals. Moreover, toxicity could be increased by microsomal metabolization of certain PAHs (metabolic activation). In the case of crude oil spills, PAHs are one of the main reasons of health and reproductive problems in water-living organisms.

Our objective was to compare the pyrene metabolism pathways of two Emydidae and one Trionychidae freshwater turtle species. As these turtles are closely related to more endangered species with similar metabolic patterns, this study also helps to characterize the dangers of PAH exposure on threatened turtle populations.

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Red-EARED sliders (*Trachemys scripta elegans*, n = 2), Chinese pond turtles (*Mauremys reevesii*, n = 3) and Chinese softshell turtles (*Pelodiscus sinensis*, n = 2) were used in the experiments. After 24 hours of pre-exposure fasting, the animals were moved in a container filled up with adequate volume of distillated water (calculated on weight and volume of the animal). The exposure occurred through oral feeding (4 mg/kg pyrene, dissolved in corn oil) or, if oral feeding was not feasible, through water (250 μg/l). Since in preliminary studies the two exposure routes provided a similar metabolic pattern, we considered the two exposure method interchangeable.

Exposed water was filtered with a glass filter (GF/C; Whatman) and passed through a solid-phase cartridge (PS@Liq; Showadenko) finally eluded with 10 ml of 70% (v/v) methanol. Pyrene metabolites were determined by HPLC (20A series; Shimadzu) with FD (RF-10AXL; Shimadzu) equipped with an ODS column (ODS-120T; Tosoh). We followed the HPLC method of Ueda *et al*. (2011) with a slight modification. Deconjugation was performed to determine the nature of metabolites.

Liver subcellular fractions from Chinese pond turtles and Chinese softshell turtles were isolated from liver samples using protocols described elsewhere[3] with a slight modification: sequential centrifugation was performed at 20,000 g for 30 min and then at 100,000 g for 90 min. Protein concentrations of each fraction were measured using the BCA protein assay reagent kit (PIERCE).

Uridine 5'-diphospho-glucuronosyltransferase (UGT) and sulfotransferase (SULT) activity were measured using the method described by Ueda *et al*.

Distinct peak patterns were measured for each species. Pyrene-1-sulfate was the main metabolite in all three species. In the case of the red-EARED sliders, 1-hydroxypyrene was measured. Both Chinese pond turtles and Chinese softshell turtles produced pyrenediol-sulfate.

Measured UGT and SULT activities suggests that low affinity of UGTs to hydroxy pyrene combined with the low reaction activity might be the reason for low glucuronidation. Regarding SULT, both investigated species showed similar sulfation activity, the activity curves indicating substrate inhibition.

Our study showed that sulfate conjugation is the dominant phase II metabolic pathway in the three examined species. To understand how these species cope with long-time exposure to contaminants, further investigaton of the first phase of xenobiotic metabolism and the exposure route is needed.

References

1. Irvin, L. and Irwin, K. 2006. Global Threats Affecting the Status of Reptile Populations. In: *Toxicology of reptiles*, pp. 9–34, CRC Press, Taylor & Francis Group, Boca Raton.
2. Todd, B. D., Willson, J. D. and Whitfield Gibbons, J. 2010. The Global Status of Reptiles and Causes of Their Decline. In: *Ecotoxicology of Amphibians and Reptiles*, 2nd Ed., pp. 47–68 SETAC Press, Pensacola
3. Ueda, H., Ikenaka, Y., Nakayama, M. M. S., Tanaka-Ueno, T., Ishizuka, M., 2011 Phase-II conjugation ability for PAH metabolism in amphibians: characteristics and inter-species differences. Aquat. Toxicol. 105, 337–343

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NOTE

Metal contaminated soil from mining area caused metal accumulation and biological responses in rats

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Abstract

In order to assess the effects of metal contamination on wildlife, we collected wild black rats (*Rattus* sp.) from mining areas (Kabwe and Chingola) and a control area (Lusaka) in Zambia and compared metal and metallothionein (MT) levels in their tissues. Furthermore, we exposed metal-contaminated soil from Kabwe to laboratory Wistar rats (*Rattus norvegicus*) for one year in order to determine the accumulation factors and effects of metals caused by soil exposure. Results of both the field and laboratory studies suggested that metal-contaminated soil caused accumulation and biological responses such as elevation of MT-2 mRNA expression levels in rats.

Key Words: biological responses, metal accumulation, rat

Environmental pollution due to rapid economic progress has had serious adverse effects on fish, food animals, wild animals, and humans in African countries[4]. Due to abundant mineral resources such as Pb, Zn, Cu, and Co, as well as Cd, which is obtained as a by-product through mining/smelting activities of these metals, metal pollution is one of the most serious problems in Zambia[1,2]. In order to assess the effects of metal contamination on wildlife, we collected wild black rats (*Rattus* sp.) from mining areas (Kabwe and Chingola) and a control area (Lusaka) in Zambia and compared metal and metallothionein (MT) levels in their tissues. Furthermore, we exposed metal-contaminated soil from Kabwe to laboratory Wistar rats (*Rattus norvegicus*) for one year in order to determine accumulation factors and effects of metals resulting from soil exposure.

In the field study, wild rats were collected in

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residential areas, commercial areas, and farms in Kabwe (n = 33) and Chingola (n = 13). In Lusaka, 18 rats were captured at the University of Zambia. Roadside soil samples were also collected from the three sites. We used the same method as in our previous study[1] for metal extraction and measurement in the soil and rat tissue samples. Concentrations of Pb, Cd, Zn, Cu, and Co in soil as well as the liver, kidney, and brain of rats were measured by atomic absorption spectrophotometry. Since MT-1 and MT-2 mRNA expression levels were used to assess biological responses for metal exposure, mRNA expression levels of these genes were measured by reverse transcription real-time PCR. The method for measurement of mRNA expressions was described in our previous study[3]. Treatment of all animals was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

In the laboratory soil exposure study, male Wistar rats were divided into three groups (ten rats/group) by type of soil exposure: 1) without soil exposure control, 2) soil containing low metal levels (Pb: 75 mg/kg, Cd: 0.4 mg/kg), and 3) soil containing high metal levels (Pb: 3,757 mg/kg, Cd: 6 mg/kg). After one year of exposure, we measured metal and MT levels in various tissues of rats using the same methods in the field study.

In the field study, high concentrations of Pb, Cd, and Zn were detected in the soil from Kabwe, while high concentrations of Cu, Co, Cd, and Pb were recorded in soil samples from Chingola. Metal concentrations in the soil from Lusaka were generally lower compared to those from Chingola and Kabwe. Wild black rats in Kabwe accumulated significantly higher concentrations of Pb and Cd in various organs than rats from Lusaka. In Chingola, significantly higher concentrations of Cu, Co, Pb, and Cd were accumulated in wild black rats than in rats from Lusaka. These results were in accordance with metal accumulation patterns in soil. MT-1 and MT-2 mRNA expression levels in wild black rats from Kabwe were significantly higher than those in rats from Lusaka.

In the laboratory soil exposure study, rats in group 3 accumulated significantly high concentrations of Pb and Cd in the liver, kidney, lung, brain, and tibia as compared to rats in groups 1 and 2. A higher accumulation of Cd as compared to Pb was observed in the liver, kidney, brain, and lung, while Pb was higher in the tibia. In addition, MT-2 mRNA expression levels were significantly higher in the kidney of group 3, and this was consistent with the observation in the field study.

In conclusion, both the field and laboratory studies suggested that metal-contaminated soil caused accumulation and biological responses such as elevated MT-2 mRNA expression levels in rats.

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References

1) Ikenaka, Y., Nakayama, M. M. S., Muzandu, K., Choongo, K., Mizuno, N., Ishizuka, M. 2010. Heavy metal contamination of soil and sediment in Zambia. *African. J. Environ. Sci. Technol,* **4**(11): 729–739.
2) Nakayama, M. M. S., Ikenaka, Y., Hamada, K., Muzandu, K., Choongo, K., Teraoka, H., Mizuno, N., & Ishizuka, M. 2011. Metal and metalloid contamination in roadside soil and wild rats around a Pb-Zn mine in Kabwe, Zambia. *Environ. Pollut,* **159**: 175–181.
3) Nakayama, M. M. S., Ikenaka, Y., Hamada, K., Muzandu, K., Choongo, K., Yabe,

J., Umemura, T., Ishizuka, M. 2012. Accumulation and biological effects of metals in wild rats in mining areas of Zambia. *Environ. Monit. Assess*, DOI 10.1007/s10661–012–2912–6, In press.

4) Yabe, J., Ishizuka, M., Umemura, T. 2010. Current levels of heavy pollution in Africa. *J. Vet. Med. Sci*, **72** (10): 1257–1263.

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NOTE

In vitro diazepam metabolism in horses

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Abstract

There is little information about drug metabolism and pharmacokinetics in horses. Therefore, it is necessary to characterize the profiles of drug metabolites for the safe use of drugs. In this study, we focused on cytochrome P450 enzymes (CYPs), which represent an important enzyme group to determine pharmacological effects of drugs. We chose diazepam as the drug of choice for this study. The aim of this study was to elucidate the metabolic pathway of diazepam in horses in comparison with rats, and to clarify CYP subfamilies responsible for diazepam metabolism in horses. Our results showed temazepam was the major diazepam metabolite produced from microsomal reactions in horse liver, but horses produced drastically less p-hydroxydiazepam as compared with rats. Furthermore, CYP3A was a major contributor from the CYP subfamily of temazepam production.

Key Words: CYP3A; diazepam; horse

Horses have a long history as domestic animals, and even now, horses play important roles in society, including use for racing, companionship, and meat production. Equine medical care has been advancing, and many drugs are used in therapy. Species diversity exists in terms of drug metabolism, and such differences cause variable pharmacological effects. However, there is little information about drug metabolism and disposition in horses as compared to other animal species[6]. Therefore, it is necessary to characterize the profiles of drug metabolites for the safe use of drugs in horses. In this study, we focused on cytochrome P450 enzymes (CYPs), which represent an important enzyme group to determine pharmacological effects of drugs.

Diazepam is in widespread clinical use in horses as anesthetics, sedatives, ataractics, and anticonvulsants[2]. Diazepam is metabolized largely in the liver, and CYP is the main enzyme for the metabolism of diazepam. Major metabolites are desmethyldiazepam (nordiazepam), temazepam, and oxazepam in horses[7].

In rats, *p*-hydroxydiazepam is also a major metabolite. However, in horses, the CYP family that is involved in diazepam metabolism has not yet been clarified. In this study, we measured the

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metabolizing activity of diazepam by using horse liver microsomes, and investigated the role of CYP enzymes in such metabolism by using CYP inhibitors. We focused on CYP1A, 2C, 2D, and 3A, which are involved in diazepam metabolism in humans and rats.

Identification of CYP enzymes related to diazepam metabolism allows the prediction of drug interactions with other medications, which is useful knowledge for clinical practice.

In this study, we used thoroughbred horses (*Equus caballus*) (n = 5) and Sprague-Dawley (SD) rats (n = 5). All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. All animals were male and aged 7 to 9 years old in horses and 13 to 16 months old in rats. Liver microsomes from these animals were prepared according to the methods described by Omura and Sato (1964)[3]. The microsomes were frozen in liquid nitrogen and kept at −80°C until use. Microsomal protein concentrations were determined by the spectrophotometric method described by Lowry *et al*. (1951)[1].

The assay procedure for diazepam metabolism was in accordance with the method described by Saito *et al*. (2003)[4]. Nitrazepam was added as an internal standard to the mixture. The samples were analyzed by high-performance liquid chromatography-mass spectrometry (LC-MS, Shimadzu, LCMS-8030). Enzyme kinetic parameters (Km and Vmax) were analyzed according to the Michaelis-Menten equation, using a computer-assisted nonlinear regression program (GraphPad Prism5).

In this experiment, we used chemical inhibitors and antibodies. Ketoconazole, α-naphthoflavone, sulfaphenazole, omeprazole, and quinidine were used as chemical inhibitors of CYP3A, 1A, 2C9, 2C19, and 2D, respectively. Anti-rat CYP3A2 antibody was also used.

From our *in vitro* experiment for diazepam metabolism, temazepam was produced in the greatest quantities, followed by nordiazepam in both horses and rats. Production of oxazepam was low in both species. Species differences existed for p-hydroxydiazepam production: in horses, the production of p-hydroxydiazepam was low, and horses had a lower *Vmax*/*Km* compared with rats. This indicates that horses had a lower ability to metabolize diazepam as compared with rats. In rats, it was reported that CYP3A2 was involved in diazepam metabolism and was responsible for temazepam production and oxazepam produced from nordiazepam[5]. CYP2C11 and CYP2D4 were also reportedly responsible for nordiazepam production and oxazepam produced from temazepam, while CYP2D3 was responsible for p-hydroxydiazepam production[5].

In vivo, it was reported that diazepam metabolites were detected from the urine of horses[2]. According to that report, nordiazepam was the first metabolite detected in urine, and temazepam was detected after a much longer time. On the other hand, the total amounts of detected nordiazepam and temazepam were not so different[2]. Compared with our results, temazepam was produced in greater quantities compared to nordiazepam in microsomes, but, in urine, nordiazepam was produced at much greater levels compared to temazepam. Differences in the metabolizing rate from nordiazepam and temazepam to oxazepam and the conjugation speed of glucuronic acid may be causal factors explaining the differences *in vivo* and *in vitro*.

In our inhibition assay of diazepam metabolism, reaction rates were decreased by adding ketoconazole, while the addition of α-naphthoflavone, sulfaphenazole, quinidine, and omeprazole only slightly inhibited the CYP reaction.

Our results suggested that CYP3A was the main enzyme responsible for diazepam metabolism in horses, as previously reported in humans and rats. CYP3A catalyzes many reactions involved in drug metabolism, including metabolism of antibiotics. Thus, drug-drug interactions must be considered when diazepam is used for treatment in horses.

Recently, seven CYP3A isoforms, i.e., CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96, CYP3A97, and CYP129, have been isolated from the horse genome[8]. It was reported that CYP3A89, CYP3A94, CYP3A96, and CYP3A97 were highly expressed in the liver[8]. In future studies, we aim to clarify the role of each isoform that is involved in diazepam metabolism.

References

1) Lowry O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin-phenol reagent. *J Biol Chem* 1951; **193**: 265–275
2) Marland, A., Sarkar, P., Leavitt, R. 1999. The Urinary Elimination Profiles of Diazepam and Its Metabolites, Nordiazepam, Temazepam, and Oxazepam, in the Equine after a 10-mg Intramuscular Dose. *J Anal Toxicol* **23**(1): 29–34.
3) Omura, T. and Sato, R. 1964. The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **293**: 2370–2378
4) Saito, K., Kim, H. S, Sakai N, Ishizuka M, Kazusaka A, and Fujita S. 2004. Polymorphism in diazepam metabolism in Wistar rats. *J Pharm Sci* **93**: 1271–1278
5) Sakai, N. and Ishizuka, M. 2009. Impact of rat P450 genetic polymorphism on diazepam metabolism. *Expert Opin. Drud Metab. Toxicol* **5**(11): 1421–1433
6) Scarth, J. P., Teale, P., Kuuranne, T. 2010. Drug metabolism in the horse: a review. *Drug Testing and Analysis* **3**(1): 19–53
7) Shini, S. 2000. A review of diazepam and its use in the horse. *Journal of Equine Veterinary Science* **20**(7): 443–449
8) Tydén, E., Löfgren, M., Pegolo, S., Capolongo, F., Tjälve, H., Larsson, P. 2012. *Journal of Veterinary Pharmacology and Therapeutics*. **10**. 1111/j: 1365–2885