行政院國家科學委員會專題研究計畫　成果報告

探討赭麴毒素在咖啡食品中之含量以及對人類細胞基因表達之影響

計畫類別：個別型計畫
計畫編號：NSC94-2313-B-040-004-
執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：中山醫學大學生物醫學科學學系

計畫主持人：余豐益

計畫參與人員：曹子杰

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 95 年 10 月 31 日

行政院國家科學委員會專題研究計畫成果報告

探討赭麴毒素在咖啡食品中之含量以及對人類細胞基因表達之影響

Evaluation of ochratoxin A occurrence in coffee products and its effects on gene expression and protein phosphorylation in human cells

計畫編號：NSC 94-2313-B-040-004

執行期限：94 年 8 月 1 日至 95 年 7 月 31 日

主持人：余豐益　　中山醫學大學生物醫學系

一、中文摘要

本文主要以酵素免疫分析法分析 20 不同的農產品中赭麴毒素的含量，其中有 12 個樣品遭受到 16 到 160 ng/mL 之污染。樣品中的赭麴毒素亦經由高效液相層析法(High performance liquid chromatography)來加以分析確認。為了確認赭麴毒素的致毒機制，西方點墨法被用來檢測不同濃度赭麴毒素處理過的 HEK 293 and MDCK 細胞中 MAPK 的活性，MAPKs (主要包括 ERK, JNK, p38 等三種蛋白質)的訊號傳遞途徑會受到各種外在刺激而活化，其中包括了氧化逆境(oxidative stress)、DNA damage、cytokines 和 apoptosis 等，結果顯示赭麴毒素對細胞的刺激和毒性並不是經由 ERK1/2 或 p38 活化來引起訊號傳遞。此外為了進一步了解赭麴毒素處理對 HEK293 細胞基因造成何種影響, 基因微陣列分析被用來檢測赭麴毒素對 HEK293 細胞基因的表達情形，此項資料仍在整理中。

關鍵詞：赭麴毒素、高效液相層析法、MAPK

Abstract

Analysis of OTA with ELISA in various agricultural commodities showed that 12 of the 20 examined samples were contaminated with OTA at levels from 16 to 160 ng/g. The efficacy of cdELISA was also confirmed by the high-performance liquid chromatography method. Treatment of various concentration of OTA from 5 to 25 μM. A significant decrease to 60% in cell viability was observed when HEK 293 and MDCK cultures were incubated with OTA for 24 h at a concentration OTA 5 μM and up. Whether ochratoxin can activate the protein phosphorylation/dephosphorylation in HEK 293 cell with the application of western blotting techniques. The results showed that treatment of various concentration of OTA did not induce ERK1/2 and p38 phosphorylation. The cDNA microarray was also used to study the effects of ochratoxin A on gene expression profile. The gene expression profiles are studied right now.

Keywords: Protein phosphorylation, ELISA, OTA, high performance liquid chromatography

二、緣由與目的

Ochratoxin A (OTA)**(Fig.1.)** is a naturally occurring toxic metabolite produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum*. It has been found as a common contaminant of cereals or in other products like coffee beans, nuts, wine, and animal organs (*1-3*). Numerous studies have revealed the role of OTA as a major causative factor in mycotoxic porcine nephropathy in many European countries (*4, 5*). Toxicological studies indicate that OTA is a teratogenic, mutagenic and carcinogenic mycotoxin, which is generally absorbed from the gastrointestinal tract in animals and has strong toxic effects on their livers and kidneys. (*1, 2, 4-7*). Although acute renal failure due to inhalation of OTA in human has been rarely reported (*8*), OTA, with a long half-life of 840 h in human blood, is frequently found at high levels in serum samples obtained from people living in regions where Balkan Endemic Nephropathy occurs. OTA is also associated with an increased incidence of tumors of the upper urinary tract in human (*9-11*). The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). The European Union has also enacted a regulatory limit for the levels of OTA in cereals (5 μg/kg), roasted coffee (5 μg/kg) and instant coffee (10 μg/kg) (*12,13*).

Development of immunochemical approaches has led to more simple and rapid methods to monitor and quantify OTA in contaminated food and feed. Although several groups have developed immunoassays for OTA detection (*19-24*), most of the assays rely on the competitive indirect ELISA (ciELISA) in which OTA-protein conjugates were coated onto microplates as solid phase. Compared with the competitive direct ELISA (cdELISA), the ciELISA is more time-consuming. To effectively analyze OTA levels in various agricultural commodities in the present study, a sensitive cdELISA was used to analyze 20 different samples. On the other hand, Mitogen-activated protein kinases (MAPKs) are important signal-transducing enzymes connecting cell surface receptors to critical regulatory targets within cells. Due to the widespread nature of fungal species, OTA is considered unavoidable contaminants in foods and feeds. Some mycotoxin, including FB1, have been shown to induce MAPK activation in various models. Whether the OTA can activate the MAPK pathway or not were also carried out in the following experiment.

三、Result

Cytotoxicity effects of OTA on HEK 293.

The cytotoxic effects of OTA on the HEK 293 as measured by the tetrazolium dye-based MTT assay are shown in **Fig.1**. A significant decrease to 60% in cell viability was observed when HEK 293 cultures were incubated with OTA for 24 h at a concentration OTA 5 μM and up. After 48 h of treatment, OTA at 5 and 10 μM caused a marked decrease in overall viability of the cells to 70 and 30% of control level. Treatment of OTA at a concentration of 25 μM for 48 and 72 hr caused a dramatic decrease to in overall viability of the cells to 20% of control.

Cytotoxicity effects of OTA on MDCK

The cytotoxic effects of OTA on the MDCK as measured by the tetrazolium dye-based MTT assay are shown in **Fig.2**. The MDCK cells are more susceptible to OTA treatment. A significant decrease to 40% in cell viability was observed when HEK 293 cultures were incubated with OTA for 24 h at a concentration OTA 10 and 25 μM. After 48 h of treatment, OTA at 5, 10 and 25 μM caused a marked decrease in overall viability of the cells to 10% of control level. Treatment of OTA at a concentration of 5 μM for 48 and 72 hr caused a dramatic decrease to in overall viability of the cells to 4% of control.

Analysis of OTA in Various Agricultural Commodities with cdELISA.

Twenty samples were collected from local food stores and subjected to cdELISA for OTA determination; the results are presented in **Table 1.** Twelve of the 20 examined samples were found to be OTA or OTA analogs positive with levels ranged from 16 to 160 ng/g. Among eight examined corn samples, sample 1 had the highest level of OTA at 63 ng/g, but OTA in samples 3, 4 and 8 were below the detection limit. Buckwheat sample 10 was found to have the highest level of OTA at 144 ng/g among four selected cereal samples. In addition, bean samples 13 and 16 had OTA levels at 74 and 43 ng/g, respectively. One of three examined coffee samples contained 160 ng/g of OTA, but the other two were free of OTA.

Effects on OTA on ERK1/2 activation in HEK 293 cells

The ability of OTA to activate ERK1/2 was investigated by exposing HEK 293 to various concentrations of OTA in medium containing only 1% serum. The ERK1/2 was determined with western blotting using antibodies specific for phospho-ERK1/2. From the western blotting result, There is no significant ERK1/2 activation observed compared to solvent-treated control.

Effects on OTA on p38 phosphorylation in HEK 293 cells

The ability of OTA to activate p38 phosphorylation was also investigated by exposing HEK 293 to various concentrations of OTA in medium containing only 1% serum. The p38 phosphorylation was determined with western blotting using antibodies specific for phospho-p38. From the western blotting result, There is no significant ERK1/2 activation observed compared to solvent-treated control.

四、計畫成果自評

本研究的主要目的以酵素免疫分析法來快速檢測分析食品及及其致毒機制之探討，首先定性及定量純赭麴毒素 A 化合物對於人類胚胎腎臟細胞株細胞的毒性動力學，接著利用藉著西方點墨法探討功能性蛋白質分子磷酸化與毒性機轉之間的關係，再來利用基因微陣列方法分析赭麴毒素 A 對於細胞中基因轉錄表現及蛋白質磷酸化的影響。由結果顯示赭麴毒素 A 對人類胚胎腎臟細胞株所造成的影響應該不是經由 Mitogen-activated protein kinases (MAPKs)。藉由研究本計畫作者亦研究與腎臟毒害相關的中草藥植物源致癌毒素馬都鈴酸，目前此一毒素抗體生產與酵素免疫分析法均已經成功建立並可分析中草藥中此一毒素之含量，此一成果已經發表在已發表於 2006 年 Journal of Agricultural and Food Chemistry 54:2496-2501.

五、參考文獻

(1) IARC. Monographs on the evaluation of the carcinogenic risk of chemicals to human, Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. International Agency for Research on Cancer, 1993; Vol. 56. 489-521.

(2) Kuiper, G. T.; Scott, P. M. Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989,** *2,* 179-248.

(3) Studer-Rohr, J.; Dietrich, D. R.; Schlatter, J.; Schlatter, C. The occurrence of ochratoxin A in coffee. *Food. Chem. Toxicol.* **1995,** *33,* 341-345.

(4) Krogh, P.; Elling, F.; Friis, C.; Hald, B.; Larsen, A. E.; Lillehoj, E. B.; Madsen, A.; Mortensen, H. P.; Rasmussen, F.; Ravnskov, U. Porcine nephropathy induced by long -term ingestion of ochratoxin A. *Vet. Pathol.* **1979,** *16*, 466-475.

(5) Dwivedi, P.; Burns, R. B. The natural occurrence of ochratoxin A and its effects in poultry: A review. *World's Poult. Sci. J.* **1986,** *42*, 32-47.

(6) Creppy, E. E. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol. Lett.* **2002,** 127, 19-28.

(7) Bennett, J. W.; Klich, M. Mycotoxins *Clinic. Microbiol. Rev.* **2003***, 16,* 497-516.

(8) Di Paolo, N.; Guarnieri, A.; Garosi, G.; Sacchi, G, Mangiarotti, A. M.; Di Paolo, M. Inhaled mycotoxins lead to acute renal failure. *Nephrol. Dial. Transplant* **1994,** *4,*116-120.

(9) Petrova-Bocharova, T.; Chernozemsky, I. N.; Castegnaro M. Ochratoxin A in human blood in relation to balkan endemic nephropathy and urinary system tumors in Bulgaria. *Food Addit. Contam.* **1988,** *5*, 299-231.

(10) Stoev, S. D. The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation . *Vet. Hum. Toxicol.* **1998,** *40*, 352-360.

(11) Chernozemsky, I. N.; Stoyanov, I. S.; Petkova-Bocharova, T. K.; Nicolov, I. G.; Draganov, I. V.; Stoichev, I. I.; Tanchev, Y.; Naidenov, D.; Kalcheva, N. D. Geographic correlation between the occurrence of edemic neophropathy and urinary tract tumors in vratza district, Bulgaria. *Int. J. Cancer.* **1977,** *19*, 1-11.

(12) European commission. Commission regulation No. 472/2002, 2002, Official J. European communities, L75, 18-20.

(13) European commission regulation 123/2005 of 26 January 2005 amending EC 466/2001 as regards ochratoxin A. L25, 3-5.

(14) Blesa, J.; Berrada, H.; Soriano, J. M.; Molto, J. C.; Manes, J. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. *J. Chromatogr. A* **2004,** *1046,* 127-131.

(15) Josefsson E.; Moller, T. High pressure liquid chromatography determination of ochratoxin A and zearalenone in cereals. *J. AOAC Int.* **1979,** *62*,1165-1168.

(16) Reinhard, H.; Zimmerli, B. Reversed-phase liquid chromatographyic behavior of

mycotoxins citrinin and ochratoxin A. *J. Chromatogr. A* **1999,** *862*, 147-159.

(17) Romani, S.; Sacchetti, G..; Chaves L.; Pinnavaia, G, G.; Dalla R. M. Screening on the occurrence of ochratoxin A in green coffee beans of different origins and types, *J. Agric. Food Chem.* **2000,** *48*, 3616-3619

(18) Trucksess, M. W., Giler, J. Young, K. White, K. D., Page, S. W. Determination and survey of ochratoxin A in wheat, barley, and coffee-1997. *J. AOAC Int.* **1999,** *82*, 85-89.

(19) Barna-Vetro, I.; Solti, L.; Teren, J.; Gyongyosi, A.; Szabo, E.; Wolfling, A. Sensitive ELISA test for determination of ochratoxin A. *J. Agric. Food Chem.* **1996,** *44*, 4070-4074.

(20) Chu, F. S.; Chang, C. F; Hinsdill, R. D. Production of antibody against ochratoxin A. *Appl. Environ. Microbiol.* **1976,** *31* ,831-835.

(21) Clarke, J. R.; Marquardt, R. R.; Oosterveld, A.; Frohlich, A.; Madrid, F. J.; Dawood, M. Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibody from the Yolk of the laying hen. *J. Agric. Food Chem.* **1993,** *41*, 1784-1789.

(22) Kwak, B. Y.; Shon, D. H. Detection of ochratoxin A in agricultural commodities using enzyme-linked immunosorbent assay. *Food Sci. Biotechnol.* **2000,** *9,* 168-173.

(23) Pestka, J. J.; Steinert, R. W.; Chu, F. S. Enzyme-linked immunosorbent assay for detection of orchatoxin A in wheat. *Appl. Environ. Microbiol.* **1981,** *41,*1472-1474.

(24) Thirumala-Devi, K.; Mayo, M. A.; Reddy, G.; Reddy, S. V.; Delfosse, P.; Reddy D. V. R. Production of polyclonal antibodies against ochratoxin A and its detection in chilies by ELISA. *J. Agric. Food Chem.* **2000,** *48,* 5079-5082.

(25) Fuchs, R.; Hult, K.; Peraica, M.; Radic, B.; Plestina, R. Conversion of ochratoxin C into ochratoxin A in vivo. *Appl. Environ. Microbiol.* **1984,** *48,*41-42.

(26) Stoloff, L.; Scott, P. M. Natural poisons. In Official Methods of Analysis, 16th ed.; Williams, S., Ed.; Arlington, VA, 1995; Chapter 49, pp 37-39.

(27) Yu, F. Y.; Liu, B. H.; Chou, H. N.; Chu, F. S. Development of a sensitive ELISA for the determination of microcystins in algae. *J. Agric. Food Chem.* **2002,** *50,* 4176-4182.

(28) Xiao, H.; Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A. Improved methods for conjugating selected mycotoxins to carrier protein and dextran for immunoassay *J. Agric. Food Chem.* **1995,** *43, 2092-2097*.

(29) Yu, F. Y.; Liu, B. H.; Wu, T. S.; Chi, T. F.; Su, M. C. Development of a sensitive enzyme-linked imunosorbent assay for the determination of domoic acid in shellfish. *J. Agric. Food Chem.* **2004,** *52*, 5334-5339.

(30)Wood, G. M.; Patel, S.; Entwisle, A. C.; Boenke, A. Ochratoxin A in wheat: A second intercomparison of procedure. *Food Addit. Contam.* **1996,** *13,* 519-525.

(31) Ueno, Y.; Maki, S.; Lin, J.; Furuya, M.;Sugiura, Y.; Kawamura, O. A 4-year study of plasma ochratoxin A in a selected population in Tokyo by immunoassay and immunoaffinity column-linked HPLC. *Food Chem. Toxicol.* **1998,** *36*, 445-449.

(32) Pittet, A.; Tornare, D.; Huggett, A.; Viani, R. Liquid chromatographic determination of ochratoxin A in pure and adulterated soluble coffee using an immunoaffinity column cleanup procedure. *J. Agric. Food Chem.* **1996,** *44*, 3564-3569.

Fig 1. The cytotoxic effect of ochratoxin on Human embryonic cell H293. H293 were treated with various concentrations of ochratoxin up to 72 h. Cell viability was determined as metabolic integrity using the MTT assay and expressed as percentage of control.

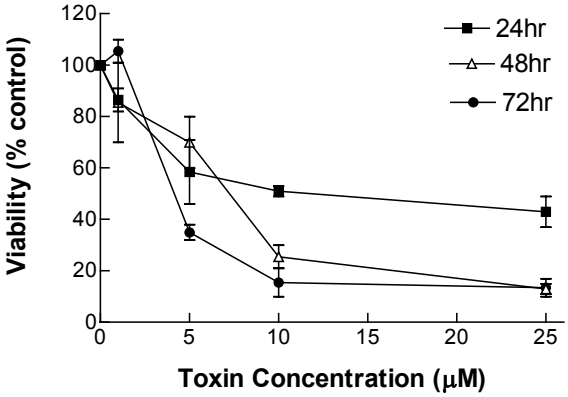

Fig. 2. The cytotoxic effect of ochratoxin on MDCK cell. MDCK cell were treated with various concentrations of ochratoxin up to 72 h. Cell viability was determined as metabolic integrity using the MTT assay and expressed as percentage of control.

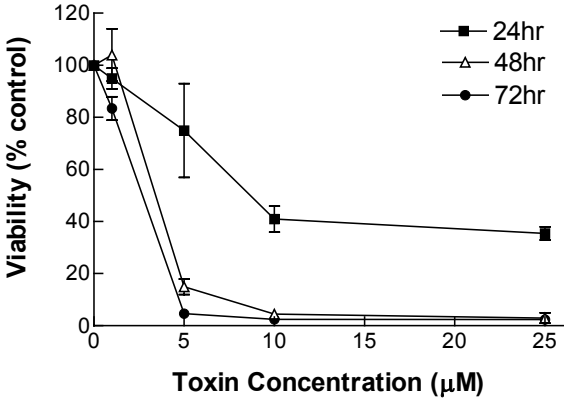

Table 1. ELISA and HPLC analysis of OTA in different agricultural commodities [a]

samples	source area	ELISA OTA and analogs (ng/g ± SD)	HPLC OTA (ng/g)
corn			
1.	Taiwan	63 ± 5	ND
2.	Taiwan	24 ± 3	*
3.	Taiwan	ND	*
4.	Imported	16 ± 3	*
5.	Imported	27 ± 4	*
6.	Taiwan	ND	*
7.	Taiwan	30 ± 3	*
8.	Taiwan	ND	*
selected cereals			
9.	Imported wheat	84 ± 4	ND
10.	Imported buckwheat	144 ± 8	60
11	Imported barley	35 ± 3	*
12	Imported barley	43 ± 4	*
bean			
13	Taiwan (black bean)	74 ± 6	ND
14	Taiwan soybean	ND	*
15	Taiwan soybean	ND	*
16	Taiwan (red bean)	43 ± 5	ND
17	Taiwan (green bean)	ND	*
coffee			
18	Imported (green coffee)	ND	ND
19	Imported (green coffee)	ND	ND
20	Imported (roasted coffee)	160 ± 9	220

[a] Each sample was extracted twice and each extract was analyzed in triplicate

[b] ND, not detected or below the limit of detection

* Do not determine.

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

探討赭麴毒素在咖啡食品中之含量以及對人類細胞基因表達之影響

計畫類別：■ 個別型計畫　　□ 整合型計畫
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計畫主持人：余豐益
共同主持人：
計畫參與人員：　曹子杰

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執行單位：中山醫學大學生物醫學系

中　華　民　國　九十五　年　十　月　三十　日

附件二

可供推廣之研發成果資料表

☐ 可申請專利　☐ 可技術移轉　　日期：95 年 10 月 31 日

國科會補助計畫	計畫名稱：探討赭麴毒素在咖啡食品中之含量以及對人類細胞基因表達之影響 計畫主持人：余豐益 計畫編號：NSC 94-2313-B-040-004-　　學門領域：食品
技術/創作名稱	
發明人/創作人	
技術說明	
可利用之產業及可開發之產品	
技術特點	
推廣及運用的價值	

※ 1.每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送　貴單位研發成果推廣單位（如技術移轉中心）。

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