Refining risk assessment of Ochratoxin A through applicable toxicologic experimentation

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Evaluación refinada del riesgo de ocratoxina a mediante experimentación toxicológica aplicada

Resumen. La Ocratoxina A es primordialmente una nefrotoxina que afecta a varias especies de animales, es también un cancerígeno renal en ratas viejas cuando el compuesto se les administra por meses. El efecto cancerígeno es la principal consideración en la evaluación de riesgo de la comida para humanos. Si la toxina es o no genotóxica por ligamiento a ADN es un factor importante para aceptarla como contaminante de alimentos. Un programa de investigación europeo busca aclarar la cuestión de la genotoxicidad a través de la detección de C^{14} ligado al ADN renal de ratas que recibieron Ocratoxina A con C^{14} de una radioactividad altamente específica. Este acercamiento se complementa con el estudio de la expresión génica de riñón de rata durante una exposición crónica, en régimen tumorigénico de Ocratoxina A, apuntando a influencias toxicológicas activas que producen carcinomas.

Palabras clave: Ocratoxina A, carcinogenicidad, genotoxicidad, aductos ADN, evaluación de riesgo.

Abstract. Ochratoxin A is primarily a nephrotoxin in many animal species but it is also a renal carcinogen in aging rats given the compound over many months. Potential carcinogenicity is the principal consideration in risk assessment for human exposure in food. Whether or not the mycotoxin is itself genotoxic by binding to DNA is an important factor in determining acceptable contamination of foodstuffs. A current European research programme seeks to clarify the question of genotoxicity through detection of any ^{14}C found bound to renal DNA of rats given ^{14}C ochratoxin A of high specific radioactivity. This approach is complemented by study of gene expression changes in rat kidney during chronic exposure to a tumourogenic regime of ochratoxin A, thereby pointing positively to active toxicologic influences leading to the carcinomas.

Key words: ochratoxin A, carcinogenicity, genotoxicity, DNA adducts, risk assessment.

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Introduction

Ochratoxin A is a very potent mycotoxin affecting many systems in animals. Probably it could affect humans but there is no case of human intoxication and morbidity clearly attributed to it. Causal association with the mysterious human disease Balkan endemic nephropathy is still hypothetical. The toxin is one of the first group that formed the field of mycotoxicology in the early 1960s, and since then has accumulated an extensive literature. Natural ochratoxicosis is best known in the pig but also may affect poultry. Additionally, laboratory rodents and cultured tissue have been the basis of much toxicological research.

In making risk assessment for humans in the absence of any direct data, reliance has been on extrapolation from natural and experimental animal data, mainly focusing on

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general affects on tubular epithelium in the kidney and in causing renal tumours in experimental rodents. Application of prudent safety factors have resulted in estimating a safe human intake amounting to about one twenty-fifth of a single grain of granulated sugar, spread evenly over one year for an average human. Consequently, the European Commission has been considering setting limits of ochratoxin A in foodstuffs at a level similar to those for aflatoxin B_1. However, it has deferred a firm decision pending assessing new data arising from a three-year collaborative research project, between seven partner laboratories in five European countries [3]. Taking a wider view, actual human risk is also uncertain for many other mycotoxins.

Being produced by *Penicillium verrucosum* in cool temperate latitudes and by several yellow- and black-spored Aspergilli in the tropics, a wide range of agricultural products are subject to contamination with ochratoxin A if the relevant fungi can grow in commodities destined for food that are not stored sufficiently dry. Mainly, contamination is post-harvest, but not exclusively so. Thus, cereals, legumes, spices, coffee and even some wines may contain trace amounts (µg/kg = parts per billion or ppb) of ochratoxin A; very occasionally larger amounts (parts per million = ppm) can occur. Consequently, with modern sophisticated analysis, traces of ochratoxin A have been found quite widely in human blood and even in breast milk. The question is "Does this matter?" Too stringent a response may unreasonably disrupt trade with serious socio-economic consequences where certain commodities are vital exports, and too little might compromise food safety.

Obtaining applicable experimental data

Applying data from toxicology experiments in animals to humans is necessarily difficult, even from quite good models such as the pig and the rat. Always, tissue culture data can be even more difficult to apply, omitting as it does that *in vivo* ingested toxins are usually routed directly into the highly complex heterogeneous enzymic matrix of the liver. There, metabolic destruction or modification for excretion, or specific activation to toxicity can occur. Some products of these transformations may be eliminated in bile, while others may move on extensively to other organs, often eliminated then via the kidney.

However, ochratoxin A is generally a very robust molecule and only a very small proportion of intake seems to be a substrate for hepatic oxidative enzymes. The toxin circulates in the blood, apparently bound quite strongly to serum proteins. Some, presumably unbound, molecules fall out of blood through the fenestrations of glomerular capillaries and are at least partly salvaged in the lower proximal tubules, partly by active transport mechanisms for the isocoumarin moiety and partly by being mistaken for phenylalanine on account of that constituting the other half of all ochratoxin molecules. Acute renal damage in the human might at worst be extremely rare, since natural occurrence of the toxin may never reach the necessary concentration. For example, in an adult rat 1 mg daily for several days in feed would hardly cause histopathological change, though the same amount given by gavage would certainly damage tubule epithelial cells [5]. Persistent mild insult by ochratoxin A in the rat slowly elicits a karyomegalic response in epithelial cells lining the P_3 segment of nephrons, where the uptake occurs. These giant aneuploid nuclei are, by definition, potential unstable preludes to malignancy. In about the last quarter of rat life, where ochratoxin A exposure has been continuous throughout most of life, renal carcinomas may arise, particularly in the males (~70% incidence). This was the finding of a huge lifetime study by the US National Toxicology Programme about 15 years ago [1]. The tumours were assumed to arise from the cortico-medullary region and some metastasised so that the secondaries may have actually caused death. This is the basis of more recent awareness of ochratoxin A as a potential risk to human health.

More specifically, clear evidence that a mycotoxin is genotoxic is a major factor in risk assessment for carcinogens. Ochratoxin A's genotoxicity is still a matter of debate; the interpretation of analytical evidence concerning adducts with DNA is crucial since there is in practice a 10-fold difference in the limit of acceptability of contamination in a food commodity, according to whether the toxin is genotoxic or not. Further, loose use of the term DNA adduct can imply to some legislators that the toxin actually binds covalently to nucleotides. Use of the term DNA adduct can easily arise through application of ^{32}P post-labelling methodology and finding even very slight evidence of DNA modifications. Meaningful modifications are likely to be rather few in number, and would need to occur consistently. Thus it can be potentially misleading to use the term DNA adduct unless and until the modifications are shown to involve all or part of the toxic molecule, in this case ochratoxin A. In the more rigid sense an adduct proves genotoxicity; in a broader sense it just raises the possibility.

Some current research objectives

Among several research approaches the current EU project addresses this dilemma for ochratoxin A in a novel way [3]. Since sophisticated analysis of foodstuffs presents the problem of the wide occurrence of ochratoxin A, why not use other sophistication to answer the question of genotoxicity? So the power of accelerator mass spectrometry, sensitive to the attomole level and more commonly applied to accurate retrospective dating by the decay of radioactive carbon, is being used. This should reveal whether or not rat renal DNA, bearing adduct-like modifications in response to ochratoxin A intoxication as revealed by post-labelling analysis, actually has the ochratoxin A molecule or a part of it attached to nucleotides. In a hypothetical positive case ^{14}C in the DNA would be above natural abundance where ^{14}C ochratoxin A is administered and DNA modifications are indeed adducts in the strict sense. To make such a study it has been necessary to make ochratoxin A radiolabelled with ^{14}C atoms as extensively as possible in the molecule, and with rather high specific radioactivity. Total radiochemical synthesis of the whole molecule is impractical, although the natural phenylalanine moiety can be replaced by ^{14}C-labeled amino acid; the peptide link can first be broken by vigorous acid hydrolysis and then reformed through classical peptide synthesis. Applying such a strategy leaves the isocoumarin moiety unlabelled, all but one atom of which is acetate-derived in the fungal biosynthesis. To overcome this incomplete labelling, shaken solid-substrate fermentation of moist shredded wheat breakfast cereal with a special strain of *Aspergillus ochraceus* [4] has been developed, in which several millicuries of ^{14}C acetate, labelled in both carbons, were added at the time when the ochratoxin synthetic pathway commenced. The fermentation was stopped a day later when labelled acetate had gone to its various metabolic fates, but before synthesis of unlabelled ochratoxin A could significantly dilute the desired product. Much prior experimental optimisation of biosynthetic dynamics was necessary before doing this, demonstrating also the wide experimental diversity necessary in the field of mycotoxicology, where so many aspects of science can interact. Some 40 mg of ochratoxin A were thus prepared, labelled exclusively in the isocoumarin moiety. Results of using this biosynthetically labelled ochratoxin A in a pilot experiment show that the ^{14}C abundance in the carbon of the renal DNA from treated rats is exactly the same as that from controls. The order of sensitivity is not less than that of the ^{32}P post-labelling evidence, implying that the small amount of modified DNA is not modified by direct binding of ochratoxin A. Greater sensitivity should come subsequently from fully radiolabelled ochratoxin A and should give unequivocal

evidence concerning the DNA modifications.

Another aspect of the current European study provides seventy male Fischer rats that have been given ochratoxin A continuously in feed since about 6 weeks old. Primarily, the purpose is ultimately to induce carcinogenesis and produce tumours, but to be able to study the processes at stages on the way. Thereby more detailed mechanistic information on the particular mode of carcinogenesis will be obtained, irrespective of whether or not the above-described genotoxicity studies point to DNA modification being an indirect consequence of ochratoxin A exposure rather than direct binding. An obvious potential model for this is the classic NTP study in which rats were given ochratoxin A by daily oral gavage 5 days a week. The highest mean daily dose of 150 mg/kg body weight over about 2 years gave the highest incidence of renal carcinoma. However, there are difficulties in applying data from gavage experiments to human risk assessment, where of course humans do not acquire mycotoxins in instant oral doses. This problem arises [5] because of the strikingly different renal histopathological and 'DNA-adduct' responses to a certain dose of ochratoxin A given either by daily gavage or mixed into feed. Consequently, in the present study ochratoxin A is also mixed into feed. Further, the NTP study continued dosing on a body weight basis throughout life, so for the latter half of life each rat at nearly 0.5 kg was receiving about 70 micrograms of ochratoxin A daily. This pattern is not a good mimic for humans, which do not all just get heavier and heavier throughout life and take in increasing amounts of food so as potentially to acquire increased amounts of a toxin. So in the present study, the ochratoxin dose in feed is set from the start at twice that of the NTP study, but held at a constant value (100 micrograms per rat) from when they are fully adult (about 0.33 kg). Thus, in the latter quarter of life the mean dose is just a little above that of the NTP study, but if the mode of administration is irrelevant a rather high incidence of renal tumours should arise in these rats.

Unfortunately there is yet no data comparing the kinetic pattern of ochratoxin A distribution when given by daily gavage or in feed, but the patterns may not be the same. Potentially, gavage could give peaks of blood concentration so that the perfused organs, and particularly the kidneys, will experience surges of toxic insult that may threaten to exceed cellular capacity for homeostasis and efficient repair. Modern concerns for experimental animal welfare, certainly in UK, limit the frequency of, for example, taking blood samples from the same rats for kinetic studies; having animals in and out of general anaesthesia adds just another experimental abnormality. However, a small study, giving a single gavage dose of several milligrams of ochratoxin A to middle-aged rats and measuring its concentration in plasma at intervals thereafter showed maximum concentration already in the first (3 h) sample. This represented less than 20% of the given dose, but surprisingly the concentration remained roughly constant over 4 days before declining. Clearly there is much yet to understand.

The lifetime rat experiment is currently entering its second year. Treated animals have grown normally and no gross abnormalities have yet been detected. The only renal histopathological change is the expected karyomegaly, becoming increasingly apparent in proximal tubules located at the renal cortico-medullary junction; some subtle evidence of DNA damage in several tissues has also been recognised. The ultimate significance of the karyomegaly is obscure and the extent of DNA damage seems so far to be within the capacity of repair mechanisms. The animal feed contains ochratoxin A at 5ppm, at least a thousand-fold higher than some of the most extreme natural contamination of any food commodity. It will be interesting to study the process of tumourigenesis in this lifetime experiment to an extent not possible in the exploratory NTP study; maybe there will be a different incidence and there could be opportunity to recognise early stages by ultrasound tomography *in vivo*. Occurrence of tumours will validate application of the gene expression data that has been gathered during the present study to probe the cryptic mechanisms of carcinogenesis that are operating in the case of ochratoxin A. cDNA array methodology for measuring changes in gene expression has advanced enormously in the past 2-3 years so that now it is possible to measure expression of 8000 genes at the same time, having snap-frozen kidney in liquid nitrogen within very few minutes of death to preserve the dynamic status of mRNAs. Patterns of significant up or down regulation should point to mechanisms, and it is hoped to have clear evidence on the contentious topic of whether or not apoptosis occurs in affected kidney tubule cells.

Need for caution

A recent publication wisely noted that demographic shifts in Europe mean that more people are exposed to natural toxicants during extended years as elderly people, who tend also to be women [2]. Modern improved general health throughout much of life in Europe results in deferred death from causes that were hardly known half a century ago. The same must also be a Pan-American phenomenon. So, could ochratoxin A be an adverse health factor other than as a potential carcinogen in the aging population? The NTP research only found carcinomas towards the natural end of life. The quoted publication above describes ochratoxin A given to old female rats, which soon showed dramatic responses. However, unfortunately many of the control rats were already dying! Thus it seems hardly sensible to experiment with the dying, with a hope of applying the findings to the elderly with a fair proportion of life ahead. Further, the ochratoxin A was given once daily by gavage in an amount of vegetable oil equivalent to an adult drinking about 300 mL all at once! A danger is that such published experimentation may be used uncritically in risk assessment.

Conclusions

Evaluation of risk assessment is not the role of the experimental scientist; it is better for it to be left to independent specialists. However, experimentalists are allowed a private view to drive enthusiastic objective exploration of mechanisms in thoughtfully-designed experiments. In its past four decades some mycotoxicological research has generated dramatic hypertoxic effects that may make interesting publications but are difficult to apply to the reality of human and animal life. Actually, studies that seek also to demonstrate negative effects may be more relevant to risk assessment for human and animal health. Further, the literature on mycotoxicology has mostly focused on exposure to single mycotoxins, and of course human experience is not like that. Ochratoxin A may have synergistic nephrotoxic interactions with both citrinin and penicillic acid, both of which are polyketides with structures similar to the isocoumarin moiety of the ochratoxins. They are also, variously, secondary metabolites of some of the same fungi that biosynthesise ochratoxin A, and of other *Penicillium* moulds that are ecologically-associated with biodeterioration of foodstuffs. Indeed, the famous Danish porcine nephropathy of the mid-20th century attributed convincingly to ochratoxin A may also have been influenced by citrinin. To be fair, risk assessment needs to take such factors also into account. Risk assessment is generally a difficult topic but it needs to be addressed fairly from the point of view of not unduly constraining international trade in agricultural products while also providing reasonable safety of human food.

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