International Orthopaedics (SICOT) (2001) 25:159–161
DOI 10.1007/s002640100240

ORIGINAL PAPER

**E. Haubruge · C. Chasseur · C. Debouck · F. Begaux
C. Suetens · F. Mathieu · V. Michel · C. Gaspar
M. Rooze · M. Hinsenkamp · P. Gillet · N. Nolard
G. Lognay**

The prevalence of mycotoxins in Kashin-Beck disease

Accepted: 16 January 2001 / Published online: 7 April 2001

Abstract Mycotoxins are naturally occurring toxic chemical compounds produced by fungi infesting agricultural crops both during their growth and storage. Such secondary metabolites, when ingested, can produce toxic syndromes in humans. As it has been suggested that mycotoxins might be involved in the development of Kashin-Beck disease (KBD), we undertook a survey of barley grains of KBD-affected families and non-affected families in that country. We found, by thin layer chromatography, a hitherto unknown metabolite of *Alternaria* sp. This was especially common on the barley grains of KBD-affected families.

Résumé Les mycotoxines sont des métabolites secondaires produits des champignons infestant les céréales au cours de la période culturale ou durant le stockage des grains. De telles substances chimiques peuvent être toxiques vis-à-vis de l'homme lorsqu'elle sont ingérées. La contamination des grains de céréales par des mycotoxines constitue toutefois une piste sérieuse pour la compréhension de l'étiologie de la maladie de Kashin-Beck. Nous avons entrepris des analyses de plusieurs mycotoxines sur des échantillons de grains d'orge provenant de familles malades et de familles non malades au Tibet. Un métabolite, encore inconnu, produit par *Alternaria* spp. a été détecté dans les échantillons de grains provenant des familles malades.

E. Haubruge (✉) · C. Gaspar
Unité de Zoologie Général et Appliquée,
Faculté Universitaire des Sciences, Agronomiques de Gembloux,
Passage des Deportes 2, 5030 Gembloux, Belgium
e-mail: haubruge.e@fsagx.ac.be

C. Chasseur · V. Michel · N. Nolard
Section de Mycologie, ISSP-Louis Pasteur, Belgium

C. Debouck · M. Rooze
Laboratoire d'Anatomie et d'Embryologie Humaine, ULB, Belgium

F. Begaux · F. Mathieu · P. Gillet
Medecins sans Frontieres, 1090 Brussels, Belgium

C. Suetens
Section d'Epidémiologie, ISSP-Louis Pasteur, Belgium

M. Hinsenkamp
Service d'Orthopédie et de Traumatologie, Hopital Erasme, ULB, Belgium

G. Lognay
Unite de Chimie Générale et Organique, FUSAGX, Belgium

Introduction

To date no consensus has been reached on the aetiology of Kashin-Beck disease (KBD), which is an endemic osteoarthritic condition found in China and other parts of Asia [13]. During the last 50 years, three hypotheses have been suggested to explain its aetiology: a deficiency in trace elements (mainly iodine and selenium), the presence of organic matter (fulvic and humic acids) in water, and the contamination of food by noxious mycotoxins [1, 12, 13].

Previous epidemiological investigations in endemic KBD areas on the Tibetan plateau have revealed agro-environmental conditions favourable to the severe infection of barley grains by fungi belonging to the Drechsera, Trichotecium and Alternaria genera [4]. In Tibet, both its high altitude and the extremely severe weather conditions affect the availability of natural resources and the use of land [7]. Nevertheless, some Tibetan barley species and cultivars are tolerant of this severe environment, and studies by archaeological researchers in the Sera Temple near Lhasa [2, 5] have revealed that these cereals constituted the staple diet of farmers for more than 64,000 years. Barley grain which is stored by 97% of Tibetan families is usually roasted and ground into 'tsampa' which is eaten with butter tea. It is also used to make 'tchang', the traditional local alcohol [8]. The barley crop is grown in the short humid summer season (June to August) and is harvested early (in August or September) before it has matured. Due to their high moisture content ranging from 15.7% to 17.5% the grains are particularly susceptible to mould [3]. After a field-drying period, the ears are beaten with flails and

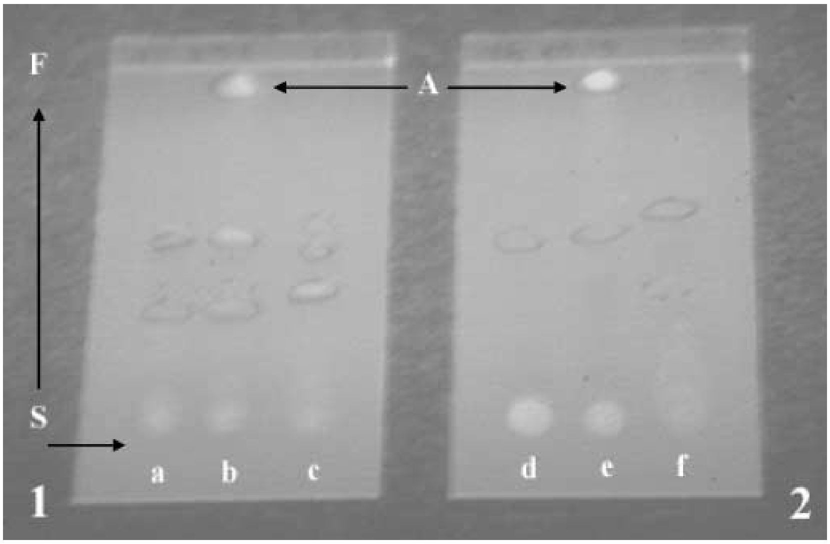

Fig. 1 Detection of mycotoxins under long-wave UV light (365 nm) after thin layer chromatography. *1*. Extracts of barley grains from non-affected families (*a*, *c*) and from a KBD-affected family (*b*). *2*. Culture extracts from *Dreschlera* sp. (*d*), *Alternaria alternata* (*e*) and *Trichothecium roseum* (*f*) isolated from Tibetan barley grains (*A* blue fluorescent spot with an *rf* of 0.96, *S* spotting area, *F* solvent front). The TLC eluent was benzene/methanol/acetic acid (90:5:5)

every year the naked grains are than inadequately stored in the same baskets, yak skin or yak hair bags.

Following our mycological examinations in Tibet, we have attempted to survey the natural occurrence of mycotoxins in the commonly used grains. Twenty-eight samples of barley collected from Tibet (P.R. China) were analysed for aflatoxins, fumonisins, ochratoxins, zearalenone (ZEA), deoxynivalenol (DON) and T2-toxin (T2) using a sensitive, competitive indirect enzyme-linked immunosorbent assay. Eighty-seven per cent of the samples were contaminated with ZEA in the range of 25–270 ppb. Seventy per cent of the samples were contaminated with T2-toxin in the range of 1–163 ppb. In contrast, the incidence of DON and aflatoxin showed low rates at 13% and 1%, respectively, with mean levels of 0–161 ppb [9]. However, no significant qualitative or quantitative differences in the incidence of contaminated samples of the studied mycotoxins were observed between KBD-affected and non-affected families. In addition, and in view of the possibility that Alternaria metabolites might be especially responsible for the occurrence of KBD in the Autonomous Region of Tibet, numerous secondary metabolites on barley grains were also studied qualitatively by thin layer chromatography (TLC) [10].

Materials and methods

Barley samples and mycotoxin extractions. Barley samples were randomly collected in October 1997 within 4 weeks of harvesting from farms located in two Tibetan villages (Rinpung and Lundrupste) of the Shigatze Prefecture. Barley grain is usually roasted and ground into ‘tsampa’ which is then eaten with butter tea. It is also used to make ‘tchang’, the traditional local alcohol [7]. Our samples were refrigerated upon arrival in the laboratory and were kept at –20°C until analysis.

Two-hundred grams of barley grains were acidified with 200 ml pH 2.0 chlorohydric acid and then ground before being twice extracted with 250 ml chloroform. These chloroform extracts were combined and concentrated on a rotary evaporator to yield an extracted homogeneous oil.

Cultural conditions and isolation of fungal metabolites. Strains of *Alternaria* ssp., *Dreschlera* spp. and *Trichotecium roseum* were isolated from barley seeds harvested from farms located in two villages (Rinpung and Lundrupste) of the Shigatze Prefecture of Tibet (P.R. China). The fungal strains (nos. 10094, 10170, 10171, 10174, 10185, 10186, 10187, 10188, 10189, 10190 and 10191) were then deposited at the IHEM collection (Institut Scientifique de la Sante Publique Louis Pasteur, Brussels, Belgium). Flasks containing 200 ml of Czapek medium were inoculated with colonies of each of the fungal strains from the Tibetan sources and then incubated at 25°C for 15 days. Fungal cultures were homogenised in NH_4HCO_3 solution (0.4%) and were shaken overnight at 4°C. The homogenates were filtered through glass wool and centrifuged at 14,926 *g* for 20 min. The supernatants were then filtered through a Millipore membrane (no. UFP1TGC24).

TLC analysis of extract. Extracts were analysed qualitatively by thin layer chromatography. They were first dissolved in chloroform before a 10-µl sample portion was spotted onto a silica gel TLC analysis (Si 460 – 40×80 mm plates). After the plate was developed with benzene/methanol/acetic acid (90:5:5) and dried, it was then studied under long-wave UV light (365 nm) and short-wave UV (254 nm).

Results and discussion

Under long-wave UV light TLC analysis of Alternaria chloroformic extracts revealed alternariol (*rf*=0.32) and alternariol monomethyl ether (*rf*=0.55). Nevertheless no obvious difference was observed between KBD-affected and non-affected families. Careful examination under long-wave UV light led to the detection of an uncommon additional compound (*rf*=0.96) which appeared as a blue fluorescent spot (Fig. 1). This molecule has been observed both in the extracts of barley grains from KBD-affected families and in the chloroformic extracts of Tibetan Alternaria strains. To the knowledge of the authors, the structure of this Alternaria metabolite remains unknown. These preliminary observations do not strictly support the hypothesis of a link between KBD and mycotoxicosis but they do constitute a promising field for further research. Additional chromatographic and spectrometric investigations are necessary to isolate and characterise this unknown molecule and further research is going to be undertaken in our laboratories.

Mycotoxins constitute a large number of naturally occurring fungal secondary metabolites with very diversified toxic effects in humans and animals. Although the 'mycotoxin problem' is an old one and intoxication from mouldy foods and feeds in humans and animals has been known for centuries, the discovery of aflatoxin as one of the most potent occurring carcinogens in the early 1960s has generated considerable research interest in this area [6]. The involvement of mycotoxin in KBD aetiology held sway in the Soviet Union at the time of the Nesterov review (1964) where the idea was proposed that mouldy grain might act selectively on joints. Experiments were conducted between 1949 and 1959 in which grain infected with *Fusarium sporotrichiella* from endemic areas when fed to rats resulted in atrophy of the growth plate and osteopenia of the skeleton but not the characteristic articular lesions of KBD. Although this hypothesis was abandoned in 1982, the possibility that fungi are an aetiological factor has again claimed attention. Indeed, in endemic KBD areas, cereals infected with *Alternaria* sp. and *Fusarium* sp. have been found often [14]. More recently in Shaanxi and Shanxi Prefectures, Luo et al. [11] have shown that *Fusarium* sp. producing known trichothecenes are involved in the fungal contamination of staple foods in endemic KBD areas. Eighty to 100% of cereal samples (maize and wheat) were found to be contaminated with nivalenol and deoxynivalenol, respectively.

Among the known mycotoxins (aflatoxins and ochratoxins) reported to be carcinogenic, several others, including some *Fusarium* sp. and *Alternaria* sp. mycotoxins (trichothecenes, tenuazonic acid) have been found to be mutagenic. Whereas extensive studies have been made on aflatoxins, little is known about the mode of action of many other fungal metabolites. The binding of mycotoxin to a specific receptor is a simple and understood mechanism of action. While zearalenone binds to the cytosolic oestrogen receptor, tremogenic mycotoxins inhibit the gamma-amino butyric acid receptors. Moreover the fungal toxic substances interfere usually with cellular machinery by affecting transduction and translation which leads to the disruption of DNA or protein synthesis.

In order to restrict mycotoxin contamination, the reduction of fungal infections in growing crops by rapid drying and correct storage of harvested crops in endemic KBD areas of Tibet is strongly recommended.

References

1. Allander E (1994) Kashin-Beck disease. An analysis of research and public health activities based on bibliography 1849–1992. Scand J Rheumatol Suppl 99
2. Avilov NI (1926) Studies on the origin of cultivated plants. Bull Appl Bot Genet Plant Breed 16:1–248
3. Chasseur C, Suetens C, Haubruge E, Mathieu F, Begaux F, Tenzin T, Nolard N (1996) Grain and flour storage conditions in rural Tibetan villages affected by Kashin-Beck disease in Lhasa Prefecture, Tibetan Autonomous Region: environmental approach. In: Milla PA (ed) Proceedings of the 8th International Congress of Bacteriology and Applied Microbiology, Jerusalem
4. Chasseur C, Suetens C, Nolard N, Begaux F, Haubruge E (1997) Fungal contamination in barley and Kashin-Beck disease in Tibet. Lancet 350:1074
5. Daniggelis E (1995) Preliminary anthropological assessment of the determinants of malnutrition among children in Tibet. Report of Tibet Child Nutrition and Collaborative Health Project, Santa Cruz
6. Goldblatt LA (1969) Aflatoxin – scientific background, control and implications. Academic Press, New York
7. Haubruge E, Broxtaux Y, Suetens C, Mathieu F, Begaux F, Durand MC, Zhu D, Michel V, Gaspar C, Claustriaux JJ, Malaisse F, Chasseur F (2000a) Ethno-ecological approach of rural environment in south-central Tibet as a support for Kashin-Beck disease prevention. II. The rural environment. Biotechnol Agricult Soc Environ (in press)
8. Haubruge E, Chasseir C, Mathieu F, Begaux F, Malaisse F, Nolard N, Gaspar C, Suetens C (2000b) La maladie de Kashin-Beck au Tibet: un probleme agro-environnemental. Cah Agricult 9:117–124
9. Haubruge E, Chasseur C, Debouck C, Suetens C, Michel V, Mathieu F, Begaux F (2000c) Occurrence of mycotoxins in stored barley in Autonomous Region of Tibet (PR China). Food Addit Contam (in press)
10. Jiajun T, Xintong H (1991) Studies on metabolic extract of *Alternaria alternata* and toxicity. J Environ Sci Health B 3:29–34
11. Luo Y, Yoshizama T, Yang JS, Zhang SY, Zhang BJ (1992) A survey of the occurrence of *Fusarium* mycotoxins in corn and wheat samples from Shaanxi and Shanxi Provinces, China. Mycotoxin Res 8:85–91
12. Moreno-Reyes R, Suetens C, Mathieu F, Begaux F, Zhu D, Rivera M, Boelaert M, Neve J, Perlmutter N, Vanderpas J (1998) Kashin-Beck osteoarthropathy in rural Tibet in relation to selenium and iodine status. N Engl J Med 339:1112–1120
13. Sokoloff L (1989) The history of Kashin-Beck disease. NY State J Med 89:343–351
14. Yang J, Lan H, Ahn M, Lu M (1983) Progress in studies on effect of fusarium toxin on Kashin-Beck disease. J Clin Endem Dis 3:155–161