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Brief communication

The targets of acetone cyanohydrin neurotoxicity in the rat are not the ones expected in an animal model of konzo ☆

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ABSTRACT

Konzo is a neurotoxic motor disease caused by excess consumption of insufficiently processed cassava. Cassava contains the cyanogenic glucoside linamarin, but konzo does not present the known pathological effects of cyanide. We hypothesized that the aglycone of linamarin, acetone cyanohydrin, may be the cause of konzo. This nitrile rapidly decomposes into cyanide and acetone, but the particular exposure and nutrition conditions involved in the emergence of konzo may favor its stabilization and subsequent acute neurotoxicity. A number of preliminary observations were used to design an experiment to test this hypothesis. In the experiment, young female Long–Evans rats were given 10 mM acetone cyanohydrin in drinking water for 2 weeks, and then 20 mM for 6 weeks. Nutrition deficits associated with konzo were modeled by providing tapioca (cassava starch) as food for the last 3 of these weeks. After this period, rats were fasted for 24 h in order to increase endogenous acetone synthesis, and then exposed to 0 (control group) or 50 μmol/kg-h of acetone cyanohydrin for 24 h (treated group) through subcutaneous osmotic minipump infusion ($n = 6$/group). Motor activity and gait were evaluated before exposure (pre-test), and 1 and 6 days after exposure. Brains ($n = 4$) were stained for neuronal degeneration by fluoro-jade B. Rats exposed to 50 μmol/kg-h of acetone cyanohydrin showed acute signs of toxicity, but no persistent motor deficits. Two animals showed fluoro-jade staining in discrete thalamic nuclei, including the paraventricular and the ventral reuniens nuclei; one also exhibited labeling of the dorsal endopiriform nucleus. Similar effects were not elicited by equimolar KCN exposure. Therefore, acetone cyanohydrin may cause selective neuronal degeneration in the rat, but the affected areas are not those expected in an animal model of konzo.

1. Introduction

The tuberous root of cassava (*Manihot esculenta*) is the main staple food in many tropical and sub-tropical regions of the world. Cassava contains nitrile derivatives of glucose, mostly linamarin (2-(beta-D-glucopyranosyloxy)-2-methylpropanenitrile), and smaller amounts of lotaustralin. These and other similar nitriles are known as cyanogenic glucosides, because they release cyanide upon metabolism [17,49]. Disruption of the cassava cells puts linamarin in contact with linamarase, a selective beta-glucosidase present in the tissue, which metabolizes the glucoside into glucose and the aglycone, acetone cyanohydrin (CAS # 75-86-5). This nitrile then decomposes spontaneously or enzymatically into acetone and HCN; therefore, chewing cassava tubers that contain high concentrations of the glucosides will lead to cyanide intoxication.

There are many varieties, or cultivars, of cassava, which all contain different concentrations of glucosides. The cultivars that are most frequently used as a staple food tend to be the most toxic, because this feature protects the crop against pests and theft [11]. Because of their cyanogenic potential, they are appropriate for consumption only after thorough processing. Several procedures are available for processing, and safe flour can be obtained from highly toxic tubers [12]. However, adequate processing may require 3 to 5 days, and the correct procedures may be ignored when socio-economic circumstances make immediate access to this staple food the overriding consideration [3,15,44,45].

Dietary use of cassava has been associated with konzo, a neurotoxic disease affecting populations in rural areas of Africa. Clinically, konzo is characterized by the abrupt onset of an isolated and symmetric spastic paraparesis which is permanent but non-progressive. Spastic gait stands out as the chief sign of the disease [2,46]. Prevalence is higher in women and children than in adult males. Epidemic outbreaks of konzo are associated with periods of agro-ecological crisis, including drought and war, but endemic occurrence has also been reported. Invariably, the disease is associated with consumption of insufficiently processed, highly toxic cassava as the main (or the only) food for a period of several weeks [3,15,20,24,44,45,47]. One striking feature of the disease is that it appears

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in an abrupt form, within days or even hours, strongly suggesting that the degeneration of a particular set of neurons is occurring in an acute time frame, despite the fact that a previous high intake of toxic cassava products for several weeks seems to be required for konzo development.

The causative agent(s) and the pathogenic mechanisms of konzo are unidentified. Insufficiently processed cassava flour contains significant amounts of linamarin, acetone cyanohydrin, and cyanide [44], and the association of exposure to cyanide and linamarin and the acute stages of konzo is well established [3,44]. However, konzo is invariably linked to a chronic pattern of cassava intake; it is a chronic disease with effects that differ markedly from those of acute cassava poisoning [1,25]. What is more, none of the known effects of acute or chronic cyanide exposure in either humans or animals, such as convulsions and delayed Parkinsonism [9,26,34] match with the singular features of konzo [2]. Linamarin is considered to have low intrinsic toxicity, besides its cyanogenic potential [10,32] and the possibility that it may be the ultimate toxic agent remains largely unexplored. One major reason for this is the lack of an affordable source of gram quantities of linamarin for animal experimentation. So far, two main non-exclusive hypotheses have been proposed. The first is that a cyanide metabolite may be the causative agent of konzo; these metabolites include cyanate [41,42], thiocyanate [39] and 2-iminothiazolidine-4-carboxylic acid [4]. The second is that deficient intake of sulphur aminoacids may compromise cyanide metabolism to thiocyanate by rhodanese [14]. The attempts to explore these hypotheses [40–42] have not provided an animal model of konzo.

The aim of this study was to explore an alternative hypothesis, namely that acetone cyanohydrin is the causative agent for konzo. This nitrile is chemically unstable in aqueous neutral or alkali solutions [18], but cassava flours associated with konzo contain significant amounts of it, due to their low pH as a consequence of lactic acid fermentation [44]. The available toxicological information for acetone cyanohydrin is scarce, but generally cyanide-like effects are reported [16,21], and exposure to this compound has been assumed to be equivalent to cyanide exposure. However, this evidence does not rule out the possibility that acetone cyanohydrin has an intrinsic neurotoxic potential. In fact, its chemical instability offers a possible explanation of the acute characteristics of the disease in association with chronic exposure: Only under particular circumstances would target organ concentrations of acetone cyanohydrin reach toxic levels; in most circumstances no cyanohydrin accumulation would occur, and its breakdown would instead lead to subtoxic cyanide exposure, as revealed by high thiocyanate serum and urine concentrations [3,44]. The hypothesized neurotoxic potential of acetone cyanohydrin would also be congruent with the fact that a number of small alkyl nitriles do cause a variety of toxic effects in selected populations of neurons and sensory cells [7,13,23,31], which may not depend on cyanide release [5,6,19].

To explore the hypothesis that acetone cyanohydrin is the causative agent in konzo, we studied the neurotoxic effects of this nitrile in the rat. Here we report a number of preliminary observations and an initial experiment evaluating its effects in an exposure model including several conditions associated with konzo such as chronic oral exposure and poor nutrition, which may be important for the expression of acetone cyanohydrin neurotoxicity and for survival after the associated cyanide exposure. Other conditions included final exposure to high doses, and fasting, which is known to increase the circulating concentrations of acetone. Acetone is one of the products of acetone cyanohydrin breakdown, and the presence of high acetone concentrations may increase the nitrile's half life.

2. Methods

2.1. Chemicals and reagents

Acetone cyanohydrin (99%) was obtained from Aldrich Química (Alcobendas, Spain), KCN (>98%) from Fluka Chemika (Buchs, Switzerland), and Fluoro-Jade B from Chemicon International (Temecula, CA, USA). Other chemicals were of analytical grade as obtained from common commercial sources.

2.2. Animals

The care and use of animals were in accordance with the Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia, and were approved by the Ethics Committee on Animal Experimentation of the University of Barcelona. Male and female Long–Evans rats (CERJ, Le-Genest-Saint-Isle, France) of different ages were used for preliminary studies. For final experiments, females of three weeks of age on receipt were used. The rats were housed two to four per cage in standard Macrolon cages (280×520×145 mm) with wood shavings as bedding at 22±2 ºC. At least 7 days were provided for acclimation before experimentation. The rats were maintained on a 12:12 L:D cycle (0700:1900 h) and given standard diet pellets (A04, U.A.R., France) *ad libitum* except when otherwise indicated.

For histology, rats were anesthetized with 400 mg kg^{-1} chloral hydrate and transcardially perfused with 50 ml of heparinized saline followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

2.3. Dosing and experimental design

We initially explored the toxicity of acetone cyanohydrin in a variety of exposure paradigms, using small numbers of animals, 1 to 3 in each group, as detailed throughout the Results section. For dosing, acetone cyanohydrin was dissolved in acidified saline (pH 3.0–3.5, with HCl) immediately before use. Subchronic exposure through acidified drinking water was also evaluated. Finally, acetone cyanohydrin exposure was performed using osmotic pumps, with the aim of modeling the continuous exposure to acetone cyanohydrin that may result from the release of this nitrile from ingested cassava flour.

The final experiment used 12 females of the Long–Evans strain, aged 35–40 days (114–123 g) at the beginning of the study. The experimental design is illustrated in Fig. 1. As we hypothesized that konzo is caused by acute high dose acetone cyanohydrin exposure in subjects chronically exposed to the same cyanogenic nitrile, we used two groups of chronically exposed animals finally receiving ("treated") or not receiving ("control") the final dose. Thus, all rats were exposed to 10 mM acetone cyanohydrin in drinking water for 14 days, followed by 20 mM acetone cyanohydrin for 42 days. For the last 21 days in this period, the rats were deprived of standard food and instead given cassava starch (tapioca, Riera Marsa brand, Nabisco Iberia, Barcelona, Spain), with the aim of modeling the unbalanced diet that is associated with konzo development. The rats were then starved for 24 h in order to induce an increase in the concentration of acetone in the body. Osmotic pumps were subsequently implanted in the animals, delivering 0 ($n=6$) or 50 ($n=6$) μmol/kg/h of acetone cyanohydrin for 24 h. At the end of this period, the pumps were removed and the animals were returned to free access to standard food pellets. The behavior of the animals was assessed at days −8 (pre-test), 1 and 6 after the end of the pump exposure period. On each

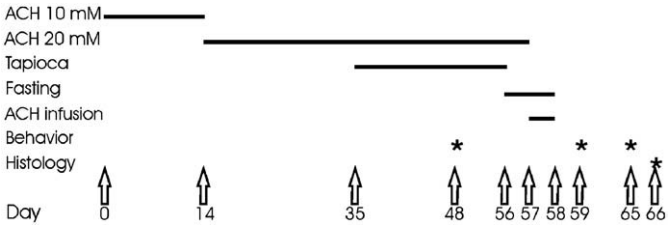

Fig. 1. Design of the experiment evaluating the neurotoxic effects of acetone cyanohydrin in the rat in an exposure model mimicking the conditions associated with human konzo. For further details, see the methods section. ACH = acetone cyanohydrin.