



Chemoprevention with chlorophyllin in individuals exposed to dietary aflatoxin

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Abstract

Because of the multiplicative interaction between dietary aflatoxins and hepatitis B virus infection in the etiology of liver cancer, efforts to reduce the consequences of either chemical or viral component are likely to have substantial public health benefit. Chlorophyllin (CHL), a water-soluble form of chlorophyll, was recently evaluated as a chemopreventive agent in a population at high risk for exposure to aflatoxin and subsequent development of hepatocellular carcinoma. CHL, which is used extensively as a food colorant and has numerous medicinal applications, is an effective anticarcinogen in experimental models including aflatoxin-induced hepatocarcinogenesis. CHL is thought to form molecular complexes with carcinogens, thereby blocking their bioavailability. In the clinical trial, administration of CHL three times a day led to a 50% reduction in the median level of urinary excretion of aflatoxin-*N*⁷-guanine compared to placebo. This excreted DNA adduct biomarker is derived from the ultimate carcinogenic metabolite of aflatoxin B₁, aflatoxin-8,9-epoxide, and is associated with increased risk of developing liver cancer in prospective epidemiologic studies. Compliance in the intervention was outstanding and no toxicities were observed. Thus, CHL has been found to be a safe and effective agent suitable for use in individuals unavoidably exposed to aflatoxins. © 2003 Elsevier Science B.V. All rights reserved.

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1. Introduction

Aflatoxins, which are naturally occurring mycotoxins found in contaminated foods such as maize, peanuts, soy sauce and fermented soybeans, have been found to be highly carcinogenic in many animal species including fish, rodents, and nonhuman primates [1]. Initially classified as a human carcinogen by the International Agency on Research in Cancer

in 1993, further epidemiological and experimental research continues to show a strong link between aflatoxin exposure and hepatocellular carcinoma (HCC) [2]. HCC is one of the most prevalent cancers worldwide with incidence rates highest in geographical regions of Africa and Asia exhibiting climatic similarities of high heat, humidity and poor food storage conditions. In the Peoples Republic of China, HCC accounts for 300,000 deaths annually and is the third leading cause of cancer mortality. The stark impact and stratification of this disease may be further illustrated by comparing the yearly incidence rate of HCC estimated for the US population of 1.5 cases

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per 100,000 to that of >100 cases per 100,000 found for some rural areas of China [3]. In these highly impacted regions, morbidity tragically equals mortality.

Early evidence linking aflatoxin exposure to HCC was based largely on estimates of aflatoxin ingestion measured in contaminated food or from dietary questionnaires. Further studies measuring urinary products (biomarkers) of aflatoxin provided a direct link to liver cancer. In the early 1990s, nested case-control studies conducted in Shanghai showed not only a significant link between aflatoxin exposure and HCC, but also a dramatic sixty-fold increase in the risk of liver cancer when aflatoxin exposure was coupled with chronic hepatitis B infection [4,5]. Additional studies in Taiwan have confirmed this striking chemical–viral interaction [6]. Thus, from a public health standpoint, major reductions in the overall incidence of HCC should result from hepatitis vaccination programs as well as efforts to reduce aflatoxin exposure. In fact, implementation of a nationwide hepatitis B vaccination program in Taiwan has been credited with reducing the incidence of liver cancer and mortality in young children by almost 50% [7].

While lowering aflatoxin contamination in foods is one approach to reducing HCC, total elimination may not be possible due to the ecology of molds that produce aflatoxins. Therefore, a different strategy of reducing dietary exposure to aflatoxins, chemoprevention, may afford a more practical and immediate means for reducing liver cancer in high-risk populations. Chemoprevention involves the use of natural or synthetic agents to block, retard, or even reverse the carcinogenic process. Hundreds of chemopreventive agents have been identified, but some of the most intriguing are natural constituents in the human diet such as those found in fruits and vegetables. Closer examination of these components has shown that they afford protection against a wide range of carcinogens [8,9]. Unfortunately, most of these compounds occur at very low levels in a nutritionally balanced diet and, in addition, they can be poorly absorbed. An exception, however, may be found in green and leafy vegetables containing the abundant, photosynthetic pigment chlorophyll and its derivative, chlorophyllin (CHL). CHL is a water-soluble mixture of sodium–copper salts of chlorophyll formed from the saponification of the relatively nonpolar, parent compound [10]. Although little is known about the

absorption of chlorophyll in humans, food samples rich in these pigments were recently shown to contain pheophytins and chlorophyllins in micellarized fractions, thereby suggesting that these compounds may indeed be absorbed in the digestive process [11]. To date, pharmacokinetic studies on CHL have not appeared in the literature; however, no known toxicities have been reported over the course of 50 years of use as an over-the-counter drug. CHL is widely marketed for a variety of dietary and medicinal uses, including food coloring agent, health food additive, accelerant in wound healing, and for the control of fecal and urinary odor in geriatric and osteomy patients [10,12]. Of all the properties attributed to CHL, the antimutagenic [13], antigenotoxic [14], and anticarcinogenic [15] activities measured against numerous carcinogens may be the most notable. Some mechanistic studies suggest that CHL acts as an “interceptor molecule” forming tight molecular complexes with planar, aromatic compounds including the potent hepatocarcinogen, aflatoxin B₁ (AFB₁) [16,17]. CHL may block the absorption of carcinogens, thereby reducing their bioavailability in target tissues ultimately leading to reduced DNA adduct formation and tumor burden. The protective properties of CHL may also be attributed to antioxidant activities [18] or the nonspecific inhibition of cytochrome P450 enzymes involved in bioactivation of carcinogens [19]. Dramatic *in vivo* examples of the anticarcinogenic potential of CHL can be seen in the inhibition of AFB₁ tumorigenesis in rainbow trout [20,21] as well as the reduction of AFB₁ disposition in rats [22]. Thus, the efficacy of CHL as an antimutagen and anticarcinogen; its potentially simple mechanistic action; its widespread, low cost availability; and the lack of reported toxicities provided a solid justification for the evaluation of CHL as a chemopreventive agent in individuals exposed to aflatoxins.

2. Study design and structure of the chlorophyllin chemoprevention trial

The chlorophyllin chemoprevention trial was a randomized, double-blinded, placebo-controlled intervention conducted in Daxin Township, Qidong, Jiangsu Province, Peoples Republic of China. Daxin is a rural farming community of approximately 40,000 residents located at the mouth of the Yangtze River,

15 km southeast of Qidong. This region has previously been identified as having an extremely high incidence of HCC with contributory factors including dietary exposure to aflatoxins and chronic hepatitis B infection. The study was designed to answer two major questions: (1) can daily administration of CHL modulate urinary and serum biomarkers of internal dose of aflatoxin? and (2) what is the feasibility of long-term administration of CHL?

Local village doctors identified potential study participants and interested volunteers were screened prior to the onset of the trial. A signed, informed consent was obtained from all participants according to the institutional and federal guidelines established by the Institutional Review Board of the Johns Hopkins Medical Institutions, the Shanghai Municipal Health Bureau and the Jiangsu Province Department of Health. Volunteers participating in the screening phase received complete physical exams and provided blood and urine samples to determine study eligibility using criteria described by Jacobson et al. [23]. Serum levels of aflatoxin–albumin adducts were also measured to assess carcinogen exposure. Those volunteers meeting all eligibility criteria including confirmed exposure to aflatoxin were invited to participate in the trial.

One hundred eighty individuals agreeing to participate in the clinical trial were randomly assigned into two equal treatment groups receiving either CHL or placebo tablets containing 100 mg of sodium copper chlorophyllin or excipient, respectively, as supplied by Rystan Inc. (Little Falls, NJ). Participants were instructed to take one tablet 20 min prior to each meal three times daily. Further details of pill distribution and compliance may be found in Egner et al. [24]. Blood and urine samples collected at 2- and 4-week intervals, respectively, throughout the 16-week intervention provided a basis for measuring toxicities and aflatoxin biomarkers. Aliquots of all collections were stored at -80°C and frozen portions were shipped to Baltimore for further analyses.

3. Compliance in the intervention and safety of chlorophyllin

Adherence to the study protocol was outstanding as only one participant withdrew during the study, and that was a result of non-medical concerns. Overall,

97% of the tablets were consumed as determined by pill counts. No toxicities or other untoward effects were reported by study participants [25]. In addition, 92 and 95% of blood and urine samples were contributed, respectively. The remarkable compliance of the study participants in the chlorophyllin chemoprevention trial, despite the thrice daily administration of CHL, directly reflects the lack of side effects of this agent. Interventions in this region with other agents, such as oltipraz, have been burdened by higher dropout rates [23,26].

4. Pharmacokinetics of chlorophyllin

At the completion of the trial when regrouping the serum samples chronologically by participant identification number, it was noted that some individuals had sera appearing to be increasingly green in color. Studies were subsequently undertaken to determine the source of the tint. Examination of the original study drug by HPLC revealed that the CHL was a mixture of several copper chlorins and derivatives of chlorophyll. The chromatographic separation of the copper chlorin components of a CHL tablet is depicted in Fig. 1. Analysis by electrospray ionization mass spectrometry (ESI/MS/MS) techniques indicated that the major chlorin found in the extracted serum was the ethyl ester of copper chlorin e_4 and that this ester was also a component of the CHL used in the clinical trial [24].

Chlorin e_4 , another component of the CHL tablet, was also detected in some serum extracts obtained from individuals receiving CHL; however, not on a routine basis. Incubation of copper chlorin e_4 ethyl ester with human serum indicated the ethyl ester was very resistant to hydrolysis by esterases and, thus, not likely to be the source of copper chlorin e_4 . Measurement of the levels of the copper chlorin e_4 ethyl ester extracted from serum samples obtained from participants receiving CHL throughout the intervention demonstrated that the ethyl ester appeared to accumulate throughout the treatment period and reached a steady-state at approximately 12 weeks into the intervention. Even though this study had not been originally designed to include a pharmacokinetic examination of CHL, it was possible to estimate the steady-state level of the ethyl ester at $3\ \mu\text{M}$ [24].

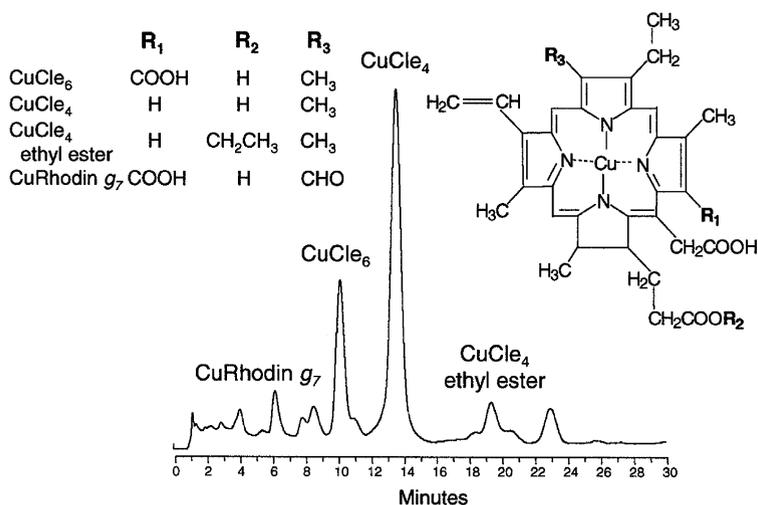


Fig. 1. HPLC tracing of the copper chlorin constituents of the CHL formulation used in the intervention. Details of the analysis can be found in [24].

It is not known whether any of the copper chlorins accumulate to equivalent or higher concentrations in cells following chronic dosing.

5. Modulation of aflatoxin biomarkers by chlorophyllin

To determine if CHL was able to modulate aflatoxin disposition, the nucleic acid adduct of AFB₁, aflatoxin-*N*⁷-guanine, was measured in urine samples collected twelve weeks into the intervention

according to the method of Walton et al. [27]. This adduct has been identified as a product derived from the ultimate carcinogenic metabolite of AFB₁, aflatoxin-8,9-epoxide [28]. A representative ESI/MS/MS analysis of urine collected from a participant is depicted in Fig. 2. The fragmentation of the parent ion, aflatoxin-*N*⁷-guanine (480.1 *m/z*), as shown in panel B, reflects cleavage into 9-hydroxy-aflatoxin and guanine ions. Following chromatographic separation, the guanine fragment (152.1 *m/z*) was quantified. Based on the maximal accumulation of the chlorin e₄ ethyl ester measured in sera after 12 weeks of

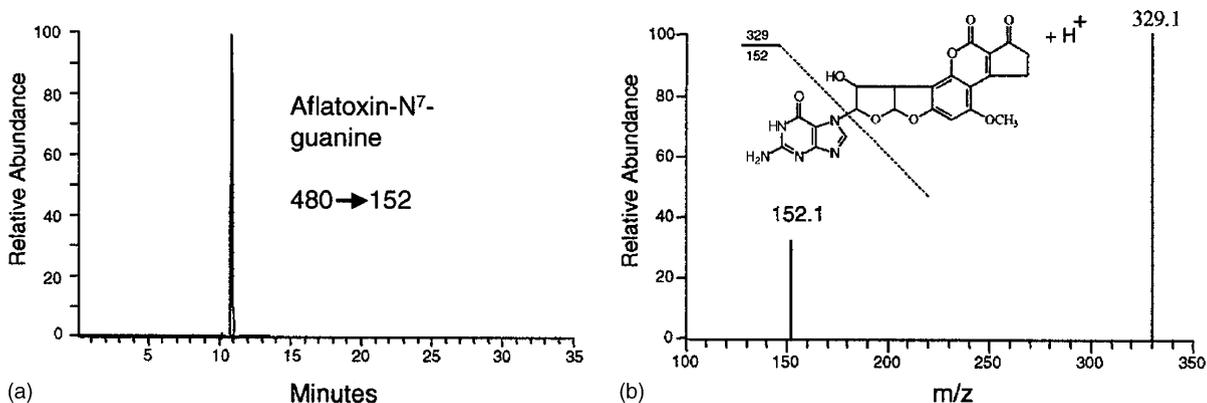


Fig. 2. Liquid chromatography–electrospray spectrometry of aflatoxin-*N*⁷-guanine isolated from the urine of a study participant.

CHL intervention, the urine samples collected at this time-point were analyzed.

Of the available 169 urine samples, aflatoxin-*N*⁷-guanine was detected (>0.02 pg/mg creatinine) in 105 (62%). For the participants randomized to the placebo and CHL arms, respectively, 63 and 61% of the urine samples collected in week 12 had detectable levels of aflatoxin-*N*⁷-guanine. Using statistical methods that allowed for a portion of those with non-detected levels to be viewed as unexposed (i.e. level = 0), it was found that all 37% of the placebo and most (38.5%) of the CHL samples with non-detectable levels were indeed unexposed. This equal distribution of unexposed participants in the two arms is an expected consequence of the randomization process. Therefore, restricting the comparisons to the 105 samples with detectable levels is appropriate. Such analysis indicated that the median urinary level of aflatoxin-*N*⁷-guanine in the participants receiving CHL was halved ($P < 0.001$) [25]. Furthermore, the 50% reduction in the excreted levels of aflatoxin-DNA adduct by CHL reflects the full magnitude of the effect of the intervention. Examination of the proportionality of the effects of CHL throughout the distribution of biomarker levels indicates that CHL had a rather uniform effect over the full range of aflatoxin exposures so that CHL reduces all levels of aflatoxin-*N*⁷-guanine to half the levels of the placebo arm. There was, however, a non-significant trend for a greater effect of CHL at the lower levels of aflatoxin exposure than at the higher levels. Such an outcome might be anticipated from an intervention that functions as an “interceptor” for aflatoxin, where the extent of protection depends upon the relative molar proportions of carcinogen and anticarcinogen.

6. Implications of aflatoxin-DNA adduct modulation by CHL

Multiple strategies for reduction and ultimate prevention of liver cancer in high-risk populations need to be developed and implemented. While there is intrinsic appeal towards primary prevention strategies, i.e. vaccination against HBV and reduced contamination of dietary staples with aflatoxins, these approaches are not without their challenges. Because transmission of HBV often occurs from mother to child, it is necessary to vaccinate high-risk populations early

in life prior to viral infection. Even (unrealistically) assuming complete coverage with a vaccine that is 100% successful, it will require more than a generation to achieve overall prevention. Attempts to reduce dietary aflatoxin exposure through improved storage conditions and diligent monitoring of levels of contamination in the developing world are strategies that will require considerable investment in the infrastructure of food production, processing and distribution. In the developed world, the consumer pays a substantial price for foods with reduced levels of mycotoxins. Such a luxury is not possible for subsistence farmers. Because of these constraints of time, money and effort, it is reasonable to consider additional, secondary approaches, towards reducing the risk of HCC and other cancers resulting from unavoidable exposures to environmental carcinogens. Such an approach is more likely to have an immediate impact on the current generation of at-risk individuals and can provide benefit until the more comprehensive and complex primary prevention strategies can be put in place.

The combined properties of versatility, availability and the strong anticarcinogenic potential of CHL in animals led to its selection for evaluation as a chemoprotective agent in a clinical trial setting. Results of this intervention indicated that thrice daily administration of 100 mg CHL led to a highly significant, 50% reduction in the excretion of a carcinogen-DNA damage biomarker, aflatoxin-*N*⁷-guanine. The important question then becomes, what, if any, is the public health significance of such an outcome?

Presence of aflatoxin-*N*⁷-guanine adducts in urine tells us several things. First, there has been an exposure. Strikingly strong dose-response relationships with this biomarker have been observed in animals and humans exposed to different amounts of aflatoxin [29,30]. Second, since these adducts are markers for promutagenic lesions, their presence indicates that damage to the genome has occurred. Indeed, over half of the HCC samples examined in the Qidong region exhibit G to T transversion mutations in codon 249 of the *p53* tumor suppressor gene [31]. These mutations are potential signatures of aflatoxins. Third, prospective, nested case-control studies that initially characterized the strong chemical-viral interaction in the etiology of HCC have clearly shown that elevated levels of this DNA adduct biomarker are specifically associated with increased risk of liver cancer

[5]. Lastly, diminished levels of aflatoxin- N^7 -guanine adducts have been associated with reduced risk of HCC in several chemoprevention studies in animals [32–34]. Interestingly, the extent of reduction of biomarker level typically underestimated the final extent of chemopreventive efficacy of the intervention [35]. Another feature of these animal bioassays is that the interventions designed to reduced aflatoxin adduct burden not only reduced the final incidence of HCC, but also significantly extended the latency period of those cancers that did arise [36].

HCC accounts for up to 10% of the adult deaths in some of the townships in the Qidong region. More-

over, mortality rates from HCC have continued to rise over the past two decades in this area. The median age of diagnosis (which is not far removed from mortality) of HCC in Qidong is 48 years. Onset of HCC is even earlier in other aflatoxin endemic regions of the world [37]. Loeb [38] has proposed that an early step in carcinogenesis is the expression of a mutator phenotype resulting from mutations in genes that normally function in the maintenance of genetic stability. Further, he has predicted “a two-fold reduction in mutation rates could prolong the time between initiation and clinical manifestations of cancer from 20 or more years to 40 or more years”. Fig. 3A shows the

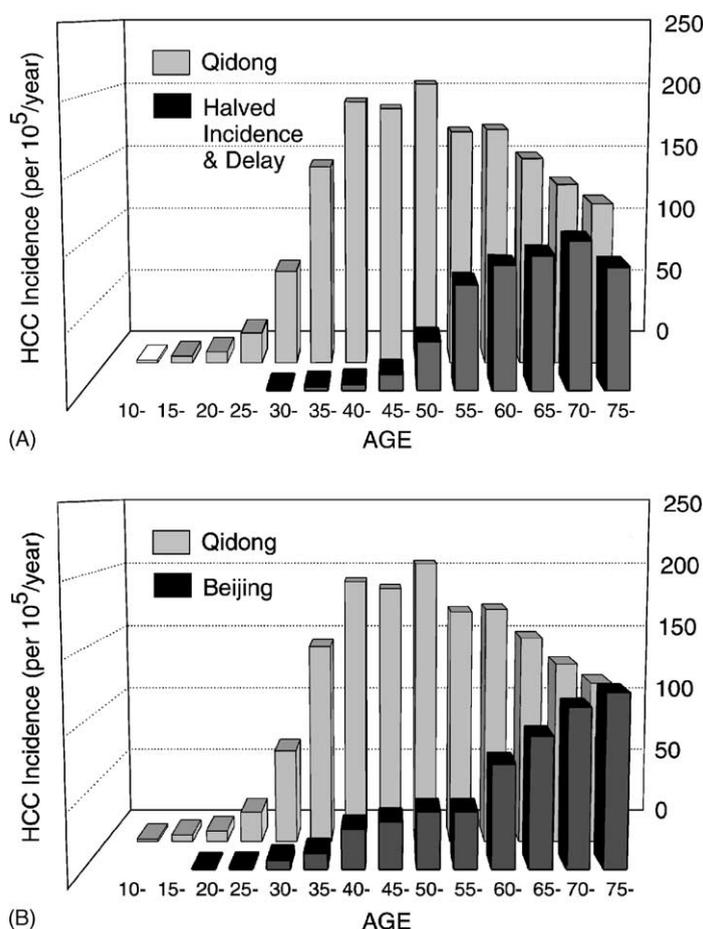


Fig. 3. Prospects for chemoprevention. (A) Age-specific incidence rate of hepatocellular carcinoma in Qidong, Jiangsu Province, Peoples Republic of China (open bars). A hypothetical distribution in which this incidence rate is both halved and delayed by two decades is overlaid (shaded bars). (B) Age-specific incidence rate of hepatocellular carcinoma in Qidong (open bars) and Beijing (solid bars). Prevalence of infection with hepatitis B virus is similar in these two regions; however, aflatoxin exposure is higher in Qidong. Data provided by Dr. Zongtang Sun, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, PRC.

age-specific incidence of HCC in residents of Qidong. Superimposed upon this histogram are the results of a simple manipulation in which incidence rates are cut in half and a 20-year lag is introduced into time of diagnosis. Both manoeuvres (reduction and delay) reflect effects observed with chemoprevention in animal models of aflatoxin-induced hepatocarcinogenesis. Remarkably, it appears that the natural experiment has been conducted. Fig. 3B shows the comparisons of the age-specific incidences of HCC in Qidong and Beijing. Prevalence rates of infection with hepatitis B virus are identical in both regions of the Peoples Republic of China; however, aflatoxin levels are considerably lower in Beijing. Thus, even in the face on viral infection, dramatic reductions in mortality from HCC apparently can be obtained by limiting aflatoxin exposures. Thus, in fact, chemoprevention strategies that reduce the genotoxic and cytotoxic effects of aflatoxins in high-risk groups can be anticipated to result in enormous reductions and substantial delay in the onset of HCC. The availability of safe, inexpensive, practical and effective protective agents, such as chlorophyllin, make transformation of this prediction into reality a public health mandate.

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