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Association between children death and consumption of *Cassia occidentalis* seeds: Clinical and experimental investigations

Gatikrushna Panigrahi [a,e], Shashikant Tiwari [b], Kausar M. Ansari [a], Rajnish K. Chaturvedi [b], Vinay K. Khanna [b], Bhushan P. Chaudhari [c], Vipin M. Vashistha [d], S. Raisuddin [e], Mukul Das [a,*]

[a] Food, Drug and Chemical Toxicology Division, Council of Scientific and Industrial Research-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, Uttar Pradesh, India
[b] Developmental Toxicology Division, CSIR-IITR, Lucknow, Uttar Pradesh, India
[c] Pathology Laboratory, CSIR-IITR, Lucknow, Uttar Pradesh, India
[d] Mangla Hospital and Research Centre, Bijnor, Uttar Pradesh, India
[e] Department of Medical Elementology and Toxicology, Jamia Hamdard, New Delhi, India

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ABSTRACT

Recently, children with high mortality rate have been observed in northern parts of India, for which the etiology is still not established, although a case control study has been linked to the consumption of *Cassia occidentalis* (CO) seeds. In the present investigation toxicity of CO seeds (0.5, 1 and 2% w/w) in diet were carried out in wistar rats. After 28 days it was observed that CO seeds caused significant increases in the serum markers viz transaminases, alkaline phosphatase and lactate dehydrogenase along with histopathological lesions in hepatic tissue. CO consumption also showed decrease in grip strength, vacuolization and myopathy of skeletal muscles along with increases in serum creatinine and creatinine phosphokinase suggesting muscular damage in animals. Neuronal damage in CO treated animals was evident by a marked increase in glial fibrilar acidic protein and decrease in β-tubulin III. The experimental findings of CO consumption showed liver, muscles and brain to be the target organs, which were similar to that of the clinical data of poisoning cases as observed in the present study. Overall, the study suggests that CO seed consumption is the main etiological factor in children population suffering from hepatomyoencephalopathy in India.

1. Introduction

The global mortality rate among children has been found to be substantially reduced from 9.6 million in 2000 to 7.6 million in 2010 (UNICEF, 2012). However, under Millennium Development Goal 4 (MDG 4) it was speculated that mortality in children should be decreased by two-third between 1990 and 2015 (UN, 2010). This is due to the fact that only few countries have achieved this goal, and much attention is required in other countries (Bhutta et al., 2010; Lozano et al., 2011). The United Nation estimates that 80 percent of the global children death in 2011 occurred in only 25 countries, and about half in only five countries including India, Nigeria, Congo, Pakistan and China (UNICEF, 2012). India alone contributes almost 30% of global children deaths under five, which is more than any other country (Bhaumic, 2013).

Over the years, success to reach the MDG 4 is mostly by controlling communicable diseases; but non-communicable diseases and injuries continue to pose a significant health concern (Srivastava and Bachani, 2011; Suryanarayana et al., 2010). Among all the causes of children death "injury" (traffic injuries, falls, drowning, poisoning and burns) alone causes 5% of global children death (Liu et al., 2012). World Health Organization (WHO) Global Burden of Disease Project suggests that among all fatal injuries, accidental poisoning in children amounts to 13% of death. In India the reported figure for fatal poisonings ranged up to 11.6% (WHO, 2008). Thus, there is growing interest that the mortality in children due to accidental poisonings can be reduced once the etiology is known (Prakash and Saxena, 2013).

During the past decade an illness in young children has been observed in several adjoining areas of western UP and Uttaranchal (India), with the involvement of muscle, liver and brain resulting in almost 70% mortality (Vashishtha et al., 2007b). The outbreak

Abbreviations: CO, *Cassia occidentalis*; HME, hepatomyoencephalopathy; CAR, Conditioned Avoidance Response; GFAP, glial fibrilar acidic protein; DAPI, 4′,6-diamidino-2-phenylindole.

* Corresponding author. Tel.: +91 522 2963826; fax: +91 522 2628227.
E-mail addresses: mditrc@rediffmail.com, mukul@iitr.res.in (M. Das).

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occurs during September to December and was earlier diagnosed as acute encephalitis of unknown viral etiology. However, it is now speculated that the illness is a hepatomyoencephalopathy (HME) syndrome, which may occur following consumption of the seeds of *Cassia occidentalis* (CO) (Vashishtha et al., 2007a), a common weed widely distributed in tropical and subtropical countries (Yadav et al., 2009). One of the earlier report suggests the number of HME cases in Saharanpur district hospital, UP, India, was recorded to be around 800 during 2002–2007 (Panwar and Kumar, 2008). Young rural children (aged 2–8 yr) belonging to poor socio-economic strata seems to be affected by CO poisoning (Vashishtha et al., 2009). WHO in its report on prevention of child injury has mentioned this aspect and urged to look into the cause of death (WHO, 2008).

Studies have shown that CO poisoning occurs in domestic animals viz horses, sheep, goats and cattle with symptoms of ataxia, muscle weakness, stubbing, and body weight loss eventually leading to death (Martin et al., 1981; Galal et al., 1985; Henson and Dollahita, 1966; Oliveira-Filho et al., 2013). Furthermore, myopathy, necrosis in hepatocytes and neuropathy has also been observed in animals intoxicated with CO (Tasaka et al., 2000; Barbosa-Ferreira et al., 2005; Calore et al., 1998). Recently, it has been reported that several cattle died in southern Brazil and North Argentina that grazed on CO weed (Carmo et al., 2011; Marin, 2010).

Literature studies suggest that there is one study related to toxicity of CO seed in rats (Barbosa-Ferreira et al., 2005), which only observed the histopathological changes in various organs. However, analysis of hematological, biochemical, neurobehavioral parameters was not performed in this study and hence exposure risk assessment to humans was not established. Furthermore, although, some toxicological studies in domestic animals have been reported in the past from other countries including Brazil, however, no systematic study in experimental animals has been conducted in Indian sub-continent. This is important from the point of view that chemical composition of plant or its parts are greatly influenced by several geographic and climatic conditions including soil characteristics. Hence, it is essential to assess the toxicity of CO from Indian context as human encounter was first suspected in this region of the globe. More importantly, the CO seed collected for toxicity assessment in this work was primarily from the area where children deaths were reported following suspected CO poisoning. Since, earlier efforts have not been made to evaluate the toxicity of CO seeds in a systematic dose dependent manner and the symptoms in domestic animals intoxicated with CO have similarity with the clinical symptoms of HME in patients, it is likely that there exist an association of CO seeds consumption with HME. Hence, the present study was designed in experimental rats to evaluate the effect of CO seeds on behavioral, hematological, biochemical, immunohistochemical and histopathological parameters and to correlate the animal experimental findings with the data of HME patients during 1998–2010 in order to decipher the etiology of the disease.

2. Materials and methods

2.1. Animal experimental study

2.1.1. Procurement of CO seeds and experimental animals

CO seeds were collected from Bijnor district of Uttar Pradesh, India, where HME in children has been reported (Vashishtha et al., 2007b). The seeds were dried in shade, grinded to fine powder and sieved to obtain a yellowish homogenous powder, which was kept for further experimental studies. Both young (60 ± 10 g) and adult (180 ± 10 g) male wistar rats were procured from the animal breeding colony of CSIR-Indian Institute of Toxicology Research (IITR), Lucknow, India and were housed in an air-conditioned room in steel cages and maintained at 25 ± 3 °C under standard laboratory conditions of light/dark cycle (12–12 h). The animals had free access to commercial pellet diet (Provini Animal Nutrition India Pvt Ltd, Bangalore, India) and water *ad libitum*. All animal handling procedures were performed according to the regulations of Institutional Animal Ethics Committee along with prior approval of animal usage (Ref. no. ITRC/IAEC/16/2009).

2.1.2. Optimization of doses for sub acute toxicity study

A pilot study was conducted to select the optimal doses of CO seeds causing toxic manifestations without any mortality. Four doses (1.0%, 2.0%, 4.0% and 8.0%) of CO seed powder in grounded diet were fed to both young and adult animals for a period of 14 and 28 days, respectively. Ten animals were taken in each group. Animals were observed for any gross behavioral and body weight changes and mortality for the above time period.

2.1.3. Sub acute toxicity study

After obtaining relevant data from Section 2.1.2, the sub acute toxicity study was conducted in adult rats following OECD guidelines (OECD 407, 1995). The detailed work plan is given in Fig. 1. In brief, animals were randomly divided into four different groups having 15 animals each. One group served as control while animals in the remaining groups were fed with CO seed powder mixed in normal diet (0.5%, 1.0% and 2.0%) for a period of 28 days. Diet consumption and body weights of animals were recorded during the treatment schedule. The total number of animals used for sub acute study was 60 (four groups each of 15 animals). After the completion of treatment schedule, 10 animals from each group were analyzed for 3 parameters e.g. grip strength, locomotive activity and memory and learning tests, in which there is no need to sacrificing the animals. Subsequently, the same 10 animals were sacrificed by anesthetic treatment and blood was collected from all the 10 animals by cardiac puncture. Blood from 5 animals were used for hematological parameter analysis immediately and serum was prepared from the remaining 5 animals, which were used for analyzing all the serum parameters. After collecting blood, the organ weight was taken from five animals from each group and subsequently these organs were fixed in formalin for histopathological analysis. Immunohistochemical analysis of brain were carried out in 5 remaining animals following blood collection. At the same time 5 animals from each group were sacrificed by cervical dislocation and further used for real time PCR analysis.

2.1.4. Locomotive behavior, grip strength and Conditioned Avoidance Response (CAR) study

Neurobehavioral studies in experimental rats were carried out in a fully computerized Actimot Monitor (TSE, Tubingen, Germany) housed in a temperature-controlled (21 ± 2 °C) room (Ali et al., 2000). In brief, rats were placed individually in the chamber and after 1 min of acclimatization, different activity scores were recorded. Each animal was subjected to one activity session of 5 min. At the end of every session, the chamber was cleaned and wiped thoroughly with 70% ethanol to remove any odor and then used again after 10 min. The locomotive markers monitored were distance travelled, resting time and movement time.

Forelimb muscle grip strengths were determined at the end of 28 days using a Grip Strength Meter (TSE, Tubingen, Germany) (Lepore et al., 2008). The test was performed by allowing the animals to grasp a thin bar attached to the force gauge. This was followed by pulling the animal away from the gauge until the forelimbs released the bar. This provides a value for the force of maximal grip strength. The force measurements were recorded in five separate trials by taking 10 animals from each group and the average values were used for analysis.

Cognitive ability (learning and memory) of the control and CO treated rats at the end of experimental schedule was evaluated by assessing a two-way conditioned avoidance behavior using a shuttle box apparatus (Columbus Instruments, Columbus, OH) as described earlier (Moreira et al., 2001). The cognitive ability of 10 rats from each group was assessed and the percentage of Conditioned Avoidance Response (CAR) was taken as the measure of cognitive ability between control and treated groups for interpretation of learning and memory.

2.1.5. Specimen collection and processing

At the end of the experimental schedule, blood was collected from the heart by cardiac puncture in dry test tubes containing sodium citrate (1:9, v/v) as an anticoagulant. A portion of the blood was allowed to clot at room temperature for 5 min followed by incubation in ice for 30 min. Serum was separated by centrifuging at 3000×*g* for 10 min and stored at −80 °C for further analysis. The remaining blood was used for the analysis of hematological parameters. After sacrificing the animals, the vital organs were removed and weighed. Some of the organs including liver, muscle and brain were fixed in 10% formalin for histopathological examination.

2.1.6. Analysis of hematological indices and serum biochemical markers

Red blood cell (RBC) count, white blood cell (WBC) count, mean corpuscular volume (MCV), hemoglobin (Hb), haemocritic rate (Hct), platelet percentage, monocyte percentage, lymphocyte and non-granulocyte percentage were examined by Automatic cell counter (MS-9, HD Consortium, India). Activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine, creatinine phosphokinase (CPK), glucose, bilirubin, total protein, albumin and albumin/globulin ratio (A/G) were estimated in serum using commercially available kits (Spinreact, Girona, Spain) following the instructions of the manufacturer.