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Aflatoxins and Hepatitis B, C Viral Associated Hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma is a serious human disease with fatal consequences. The most distressing aspect of hepatocellular carcinoma is the limited improvement in mortality (mortality rate of more than 90%). At present, the underlying molecular mechanisms are not well understood and treatment options are often of limited efficacy. This review presents our current understanding of the burden of hepatocellular carcinoma on human health, pathogenesis and pathophysiology, and molecular mechanisms associated with the disease, as well as our knowledge of the physical barriers, cellular mechanisms and molecular elements that may be targets for therapeutic interventions and/or the development of preventative measures. As the proposed findings present a major risk to public health, it is hoped that robust intervention measures will be introduced for aflatoxins monitoring and reduction in diet.

Keywords: Hepatocarcinogenesis; Hepatocellularcarcinoma; Aflatoxins; Mycotoxins; Risk factors; Pathogenesis

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of all cancer related deaths worldwide [1]. According to IARC (2012), 782,000 new cases of HCC are diagnosed per year with a mortality rate of 95% (Table 1) [2]. The overall incidence in developing countries is five times that of developed nations (513,000 vs. 110,000) [3]. In United States, the overall incidence rate of HCC has been estimated at 2.99 per 100,000 [4,5]. However, for Asian and African countries, figures are quite alarming, i.e. about 50-150 cases per 100,000 [3,6]. Another report suggests approximately 82% incidence rate in developing countries, particularly among South Asia with an annual incidence of 24 per 100,000 with much greater occurrence in the high-risk areas [7,8] (Figure 1 and Table 2). This is because these areas have higher risk factors, such as high prevalence of hepatitis B and C infections, behavioral factors like obesity, diabetes mellitus, betel nutschewing, and an important but largely ignored factor of exposure to aflatoxins [8-10].

Aflatoxins are secondary metabolites primarily synthesized by food-borne Aspergillus species. Aflatoxins commonly contaminate maize, nuts, rice, wheat, and spices like chilies, black pepper, turmeric and other food items that are consumed in raw form. Products obtained from animals that have ingested contaminated feed are also recognized as indirect source.Aflatoxins being highly lipophilic are readily absorbed through the gastrointestinal tract. They may also enter the bloodstream directly by inhalation [11]. On a global scale, approximately 4.5 billion persons living in developing countries are frequently exposed to unchecked amounts of the toxin [12] (Table 3). Consumption of aflatoxin-contaminated food has been associated with HCC [13]. According to a study conducted in 2010, there are 550,000–600,000 new HCC cases reported and out of those about 25,200–155,000 may be attributable to aflatoxin exposure [14]. The regions where most of these cases occur are sub-Saharan Africa, China, and Southeast Asia; areas where the elevated prevalence of viral hepatitis, as well as higher occurrence of aflatoxin-contaminated foods, especially the maize have been reported [14]. For example in China, reported incidence of contamination in corn was 85%, while in Pakistan, reported incidence of aflatoxin contamination inmaize was found to be 41.6% and 25% in red chillies, respectively [12,15] (Table 4). Both countries have significant prevalence of viral hepatitis infections as well [16].

There are many types of aflatoxins (B1, B2, G1, G2 and M1) but aflatoxinB1 (AFB1) has been classified as a Group 1 carcinogen (definitely carcinogenic to humans) and one of the most potent toxins associated with liver cancer [17]. AFB1 metabolites have the ability to bind with albumin and the measurement of aflatoxin-serum albumin adducts has been used to detect recent exposure in individuals [18-20]. AFB1 metabolites react with guanine residues in DNA resulting in (AFB1)-N7-guanine adducts. A Guanine to Thiamine transversion mutation in codon 249 of TP53 gene has been strongly associated with aflatoxin exposure and HCC cases [21]. The ability to modulate DNA activity by creating such lesions in the DNA may play a role in AFB1 induced carcinogenesis [22].

There is a significant synergetic effect between aflatoxin and Hepatitis B virus (HBV) infection [23]. Another study has associated aflatoxin exposure with advanced liver disease in chronic Hepatitis C patients [18]. This objective of this article is to emphasize the deleterious effects of aflatoxins-contaminated foods and to highlight the relationship between aflatoxins and viral associated hepatocellular carcinoma. As the proposed findings present a major risk to public

Region	World	More developed regions	Less developed regions
Cases	782	134	648
Deaths	746	123	622
Percentage (%)	95	92	96

Table 1: Incidence of HCC in the world. There is poor prognosis of HCC, with majority of patients expiring within 5 years. The majority of the cases are from less developed regions [2].

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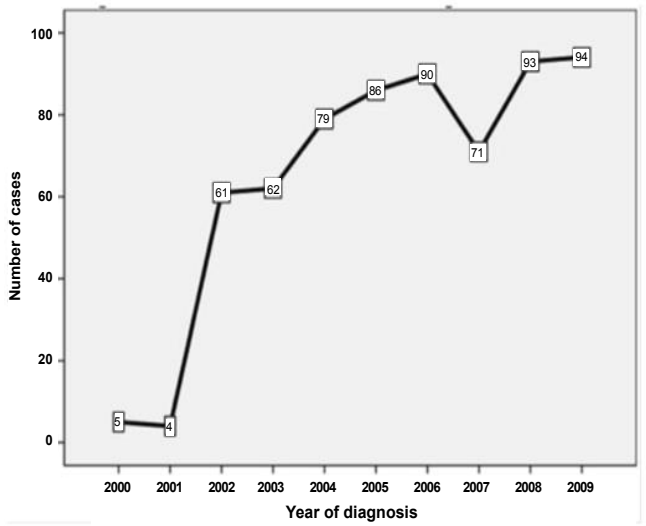

Figure 1: Number of incidence from 2000-2009.

Year	Gender	ASR for HCC/ 100,000 in Karachi
1995 - 1997	Males Females	5.7 3.7
1998 – 2002	Males Females	5.3 4.0
Year	**Gender**	**ASR for HCC/ 100,000 in Larkana**
2000 – 2002	Males Females	10.5 2.0
Year	**Gender**	**ASR for HCC/ 100,000 in Hyderabad**
1998 - 2002	Males Females	4.4 1.2
Year	**Gender**	**ASR for HCC/ 100,000 in Quetta**
1998 - 1999	Males Females	12.3 3.1
Gender		**ASR for HCC/ 100,000 in Karachi**
1995 - 1997	Males Females	5.7 3.7
1998 – 2002	Males Females	5.3 4.0
Year		**ASR for HCC/ 100,000 in Larkana**
2000 – 2002	Males Females	10.5 2.0
Year		**ASR for HCC/ 100,000 in Hyderabad**
1998 - 2002	Males Females	4.4 1.2
Year		**ASR for HCC/ 100,000 in Quetta**
1998 - 1999	Males Females	12.3 3.1

Table 2: Year wise increase in HCC cases with respect to gender in different cities of Pakistan. The male to female ratio is 3.6:1.0 [7,61]

health, it is hoped that robust intervention measures will be introduced for aflatoxin monitoring and reduction in diet.

Synergism of Aflatoxin and Hepatitis B Viral Infection in HCC

HBV and HCC

According to WHO, HBV is the second known carcinogen affecting liver after tobacco and there are approximately 350 million people chronically infected with HBV worldwide and responsible for at least 75% of HCC. In the US, Africa and Europe the prevalence of HBV among HCC cases was reported as 9%, 40% and 22%, respectively [16]. In Asia, proportion of HBsAg positive HCC cases was greater than 50% in China, Taiwan, Korea, Thailand, Vietnam, and Turkey. However, for Pakistan and India the prevalence is 30 and 45% respectively [16]. The annual risk of HCC is 0.5% for asymptomatic HBsAg carriers and 0.8% for patients with chronic hepatitis B [24]. Both HBV and HCV can directly lead to HCCreviewed in HBV is an oncogenic virus that partially contains double stranded DNA and it can directly integrate into the host genome, leading to changes in genomic function or chromosomal instability [25]. Of note, the integration of HBV DNA into the host genome is not required for its replication, however it does allow for the persistence of the viral genome within the host. Furthermore, several HBV factors have been implicated in hepatocarcinogenesis, including the HBx gene, the pre-S2/S gene and the HBV spliced protein. By contrast to HBV, HCV is a positive-stranded RNA virus. As it lacks reverse transcriptase, it cannot integrate into the host genome. Being a completely cytoplasmic replicating virus, its involvement in HCC occurs via indirect pathways through the effects of chronic inflammation and hepatocellular injury.

Food Product	Country	Levels in µg/kg	Maximum Allowed level according to commission of European Communities. (µg/kg)	Reference
Bread	Lebanon	1.12	2.0	[62]
Chocolate	Lebanon	4.33	2.0	[63]
Nuts	Lebanon	4.40	8.0	[63]
Rice, wheat, Oat, Barley, Maize	Malaysia	0.12-4.54	5.0	[64]
Peanuts	Malaysia	0.66-15.33	8.0	[64]
Rice	Iran	0.0029	5.0	[65]
Puffed Corn Snack	Iran	0.0012	8.0	[65]
Peanuts	Iran	0.00154	8.0	[65]
Bean	Iran	0.21-0.29	8.0	[66]
Maize	Iran	30	5.0	[67]
Peanuts	Iran	15	8.0	[67]
Rice	Pakistan	8.5	5.0	[68]
Corn	Pakistan	8	8.0	[68]
Corn Products	Pakistan	5.5	8.0	[68]
Dried Chili	Pakistan	Upto 90	5.0	[69]
Capsicum	Pakistan	Upto 25	5.0	[70]
Corn based products	Indonesia	5.8 – 12.4	8.0	[71]
Corn	Indonesia	119	8.0	[72]
Corn	Phillipines	49	8.0	[73]
Corn	Thailand	63	8.0	[73]
Maize	Taiwan	15	5.0	[67]
Peanuts	Taiwan	15	8.0	[67]
Rice	India	<15	5.0	[74]
Maize	India	30	5.0	[67]
Peanuts	India	30	8.0	[67]
Maize	China	40	5.0	[67]
Peanuts	China	49	8.0	[67]
Nuts and Product	Japan	0.3-128	8.0	[75]
Maize	Nepal	40	5.0	[67]
Peanuts	Nepal	40	8.0	[67]

Table 3: Aflatoxin levels in staple food products of different countries in Asia.

Sampling Region in Pakistan	Types of Mycotoxins studied	Types of Foodstuffs Analyzed	No. of Samples Analyzed	µg/kg[a]	Percentage of positive samples[b]	References
Punjab	aflatoxin B_1, total aflatoxins and ochratoxin A	rice corn corn products	68 105 102	12.08 5.47 7.85	28 14 20	[68]
36 districts of Punjab	aflatoxin M_1	milk sweet	232 138	0.252 0.58	32 78	[76]
Across Pakistan	aflatoxin B_1, B_2, G_1, G_2	paddy rice parboiled rice brown rice white rice broken rice	58 69 84 93 109	16.35 14.20 9.85 7.10 8.50	64 38 33 42 50	[40]
Punjab	Aflatxin M_1	milk, yogurt, white cheese, cheese cream, Butter	107 96 119 150 74	150.7 90.4 147.8 102.6 69.7	58 47 15 11 52	[77]
Faisalabad District Punjab	Aflatxin M_1	buffaloes milk, cows milk, goats milk, sheep milk, camels milk	55 40 30 24 20	0.013 0.014 0.002 0.002 Nil	15.8 20 nil nil nil	[78]
Punjab	Aflatoxin B_1, B_2, G_1, G_2	Longi chilies Wonder hot chilies Skyline 1 chilies	15 12 13	15.91 5.77 12.15	53 8 38	[79]
Swat Valley NWFP	Aflatoxin B_1ochratoxin A	Maize Kernels upper swat Maize Kernels lower swat	18 18	14.94 16.22	66 88	[80]
Major districts of Punjab	AflatoxinB1 Aflatoxin M1	halva pistachio almonds semolina cardamom raisins halva puri wheat powder	56 71 63 69 34 46 39 53	3.79 9.48 12.98 7.45 2.34 5.34 7.80 5.67	20 23 34 12 11 7 9 11	[81]
Punjab 4 districts (Faisalabad, Lahore, Jhang and Toba Tek Singh)	aflatoxin B_1, B_2, G_1, G_2 Ochratoxin A	<u>open market</u>: chilli sauce crushed chilli powdered chilli <u>Resturant:</u> chilli sauce crushed chilli powdered chilli	 26 29 34 24 28 29	 10.4 22.9 27.5 11.3 19.8 21.1	 12 27 35 8 32 41	[82]
NWFP and Northern area of Pakistan	aflatoxin B_1, B_2, G_1, G_2	<u>Dried fruits & nuts:</u> dried apricot, dates, dried figs, mulberries, dried raisins, apricot kernels, almonds with shells, almonds without shells, walnuts with shells, walnuts without shells, peanut with shells, peanuts without shells, pistachios with shells, pistachios without shells, pine nuts with shell,	 20 20 10 15 10 15 10 10 10 10 10 10 10 10 10	 4.55 2.5 5.81 2.22 5.05 2.65 nil 2.13 6.45 3.43 5.10 5.20 2.10 6.34 3.25	 5 nil 30 nil 10 7 nil nil 20 30 10 20 nil 20 nil	[83]
Central area of punjab	Aflatoxin M_1	Winter season & summer Raw milk UHT milk Yogurt Butter Ice cream	48 & 56 45 & 39 51 & 45 35 & 35 42 & 37	0.073 & 0.028 0.060 & 0.021 0.053 & 0.019 0.036 & 0.015 0.021 & 0.012	27 & 23 24 & 23 25 & 18 34 & 20 17 & 5	[84]

Table 4: Natural Aflatoxin occurrence in Pakistani foodstuffs. ([a]Average concentration of total aflatoxins where multiple aflatoxins analyzed, otherwise aflatoxin B_1 or M_1; [b]With respect to EU MRLs)

In this regard, several HCV proteins, including the core, envelope and nonstructural proteinsmay be involved in HCC development via oxidative stress, cell cycle control, and tumor formation.

Aflatoxin, HBV and HCC

Studies reveal that South East Asia and Africa show a significant overlap between AFB1 exposure and HBV prevalence [14]. According to a cohort study by Ming et al., even moderate levels of aflatoxin in diet almost tripled the chances of developing HCC in men infected with HBV. Another study reported 30 times greater risk of developing HCC in individuals co-exposed to both HBV and aflatoxin exposure [26]. In comparison to HCV, HBV infection appears to carry a higher risk in causing HCC with a 20-fold lower odds ratio for HCV. A mutation at 249ser-p53 has been identified as a marker to detect concurrent HBV infection and aflatoxin exposure. It was found to be present in 54% of the HBV positive HCC cases in Oidong, China [27]. While the same mutation was found to be absent in 42% matched HCC cases from Beijing, known for relatively low aflatoxin exposure [28,29]. Several epidemiological studies con¬firm that aflatoxin's cancer causing potency is much greater among HBV-positive than among HBV-negative individuals [23,27,30]. A strong testimony for the synergistic effect of aflatoxin in HBV-associated HCC comes from the fact that AFB1 is associated with a G-to-T transversion in codon 249 of the TP53 gene which changes arginine to serine. This mutation has been seen to interact with HBV X protein in altering cell proliferation and chromosome stability of liver cells [31].

Studies on animals have shown that injury to liver cells inflicted by HBV can increase the amount of cytochrome p450 in liver cells thereby inadvertently increasing aflatoxin metabolism and activation. This could also explain why children acutely infected with HBV show highest adduct levels, followed by chronic carriers as compared to uninfected children [32].

Increased risk of HCC with aflatoxin exposure and Hepatitis C Viral infection

HCV and HCC

An earlier report of the World Health Organization suggested that approximately 170 million people are chronically infected with HCV worldwide [33]. However, considerable regional variations in the prevalence of HCV infection in HCC patients is also reported. In the US, Africa and Europe, the prevalence of anti- HCVpositiveindividuals among HCC cases was 22%, 69% and 45%, respectively. In Asia, majority of HCC cases are reported from Japan with 68% sero prevalence of anti-HCV antibodies. Prevalence of HCV infection in Pakistan is 6.5%with a high proportion (45%) of anti-HCV antibodies also detected among HCC cases [16,34]. While active HCV RNA infections reported in different regions of Pakistan ranged from 3.5-4.9% [35,36].

Aflatoxin, HCV and HCC

Even though aflatoxin and HBV synergism for HCC has been reported, similar synergistic effects of aflatoxin in chronic HCV infection remain to be settled. A conclusive evidence to this effect is hindered by the fact that chronic HCV infection usually occurs later in life and persists for a long time, whereas chronic HBV infection occurs much earlier, thus it is possible that the time of overlapped exposure for aflatoxin and HCV is less significant [14]. An attempt to link HCC prevalence and geographical overlap in populations exposed to aflatoxin, and populations with a high prevalence of HCV, remained inconclusive due to variations encountered in results. For example, there was more prevalence of aflatoxin, HCV and HBV in Cameroon as compared to Gambia but the latter had higher HCC rates. Perhaps this was because of limited data available on HCV prevalence or other additional risk factors that were unaccounted for [37]. An association between HCV positive patients with Advanced Liver Disease (ALD; liver cirrhosis or HCC) and aflatoxin levels has also been reported. While ALD patients without anti-HCV positivity revealed no significant association with aflatoxin levels. Hence, it can be assumed that ALD does not cause increased aflatoxin activation. However, these studies did not look at dietary aflatoxin levels [18]. Therefore, investigations on associating dietary aflatoxin levels in HCV associated HCC are needed.

In another study in an HCV-endemic country, the rise in HCC cases was strongly linked with food and seeds contaminated with aflatoxin. The consumption of aflatoxin contaminated foods was assessed and correlated with liver biopsy grade and stage scores of chronic HCV patients. It was found that chronic HCV patients who had consumed all seven types of aflatoxin contaminated foods were positively correlated to grade 3 and stage 3 of liver disease, which is a strong precursor for development of HCC [38].

Sun et al., assessed the aflatoxin albumin adduct (AF-alb) levels for anti-HCV positive (n=36) and negative (n=406) subjects in a case control study in Taiwan. They found that anti-HCV positive subjects had less AF-alb detection rates (30.6%) as compared to anti-HCV negative subjects (41.6%). This could possibly be because of low sample size for anti-HCV positive subjects [39].

Generally, during chronic HCV infection about 10-20% of the infected population develops cirrhosis and out of those about 1-4% develop HCC. However, in Asian populations, a much higher percentage (approximately 20%) of chronic HCV infections are resulting in HCC (Figure 2). As increased prevalence of aflatoxin contaminated foods has been reported in these areas [14], this brings us to wonder whether AFB1 could be acting as a co- carcinogen. In Pakistan, rice is a major contributor to the dietary intake of aflatoxins. The total estimated amount of aflatoxin intake for average rice consumers ranged from 19.1 to 26.6 ng/kg body weight/day, which is much higher than the reference value of 1 ng/kg body weight/day [40].

Oncogene activation by HCV, could aflatoxins exacerbate it?

HCV enhances the transcription of c-myc oncogene which may contribute to the development of cancer [41]. The non-structural protein, NS4B is known to transform NIH3T3 cells in association with Ha-ras oncogene [42]. NS4B also causes upregulation of RAP1 and FYN oncogenes in HeLa cells [43].

In a study in Taiwan, aflatoxin levels were determined in serum as well as plasma of the same patients and these samples were then tested for p53 mutations and p16 methylation. These two markers are indicative of cancer development, p53 beingone of the most frequently mutated genes. A significant correlation between aflatoxin adducts, p53 mutation and p16 methylation was observed [44]. Another study has reported the ability of AFB1 epoxide form to cause mutations in human Ha-ras proto-oncogene in vitro [45].

Reactive Oxygen species (ROS) in HCV and role of aflatoxin as an agent of oxidative stress

During HCV infection, the production of ROS is enhanced as mitochondrial function is impaired, at the same time liver antioxidant system is affected leading to aggravated oxidative stress [46]. The envelope protein, E2, of HCV has been implicated in upregulation of collagen α(1) and oxidative stress in Huh7 cells [47]. Another viral

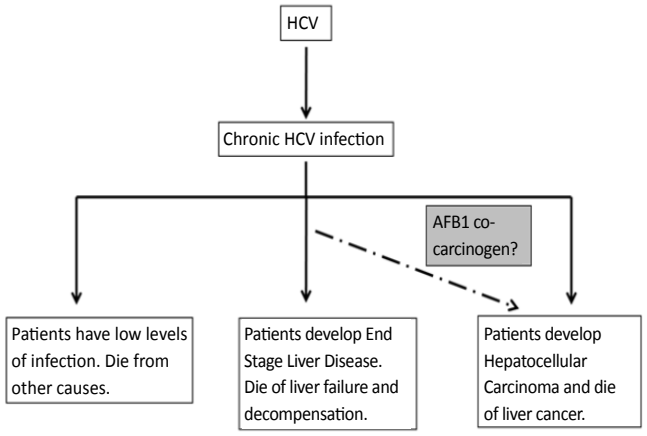

Figure 2: Details of chronic HCV infection.

protein, core protein,has been observed to interact with heat shock protein Hsp60, a stress protein chaperon. This interaction increases ROS production, which upregulates TNF-α-induced apoptotic cell deathin Huh7 cells [48].

Exposure to Aflatoxin G1 revealed induction of oxidative stress by increasing ROS generation and causing DNA double-strand breaks which was reduced upon treatment with anti-oxidants [49]. Interestingly, vitamin A, C and E exhibited a protective effect in fresh lymphocytes isolated from human blood by inhibiting ROS produced by AFB1 exposure [50]. As low as 0.05 ng/ml of AFB1 unregulated the production of ROS in polymorphonuclear leukocytes which are at the first line of defense against invading bacterial and fungal infections [51].

Immune system interference by HCV and role of aflatoxins as an immunomodulatory agent

The virus modulates a number of the host cell's defense processes to establish complete infection. Impairment of complement mediated pathwayto hinder immune activation and thereby delaying resolution of infection was observed [52]. Down regulation of complement component C9 in patients as well as in cell culture was observed and it was attributed to the core protein of HCV [52]. Using transgenic mouse models, it has been observed that nonstructural protein 5A (NS5A) of HCV impairs antiviral response and weakens interferon immune response along with cytotoxic T lymphocyte response [53].

Numerous studies have attested that AFB1 exposure can affect the immune system in a variety of ways. Secretory Immunoglobulin A in saliva of Gambian children was noted to be lower when exposed to aflatoxin contaminated food [32]. At the same time these mycotoxins have led to increased susceptibility to bacterial and parasitic infections and adversely affect acquired immunity. Human monocytes when exposed to AFB1 in vitro showed reduced microbicidal activity against Candida albicans even at low doses of 0.05-0.1 pg/mL. Both decreased phagocytosis and decreased secretion of interleukin-1, interleukin-6 and tumor necrosis factor-alpha were observed [54].

Patients suffering from HIV were tested for aflatoxin exposure via aflatoxin-albumin adducts and it was observed that those with high aflatoxin-albumin adducts had significantly low T and B cells [55]. Hendrickse et al., observed that aflatoxin contaminated-heroin addicts showed rapid progression of human immunodeficiency virus and acquired immune deficiency syndromes [56]. They attributed this accelerated rate of HIV progression to aflatoxin related immune suppression. This same synergy can be used to explain the faster rate of HIV progression in Africa when compared to Europe or the United states where aflatoxin exposure is comparatively controlled. Therefore, it is quite possible that accelerated rate of cancer development maybe be observed in those HCV patients who are consuming aflatoxin contaminated foods.

Conclusions

In order to improve food quality and to lower the aflatoxin levels in food items various practices need to be adopted including effective monitoring mechanisms from produce to products, fungal-resistant crops. At the consumer level, a change from aflatoxins-susceptible diet in highly endemic areas is another possibility. For example, recent reforms in China included changing the staple diet from maize to rice in the 1980s which actually led to reduced aflatoxin levels in sera and ultimately lower primary liver cancer cases [57]. Farmers can opt for crops that are resistant to the fungal infection or introduce fungal strains that are inefficient in producing aflatoxins. Irrigation techniques need to be improved along with drying and storage practices in order to keep the moisture content to a minimum. Testing of crops for aflatoxin contamination needs to be mandatory and cheap methods of doing so are needed. If crops have been contaminated then measures need to be introduced for decontamination and detoxification. These include food and feed processing, biochemical and microbial inactivation, chemical degradation and reduction in toxin bioavailability by selective chemisorption with clays. Physical methods may include thermal heating but due to toxin being heat stable, this has not been successful. Irradiation through radiolysis of water has shown some prospect. A last resort is always physical separation of contaminated parts by manual, mechanical or electronic means [38]. For example, in a study conducted in China, oltipraz, an antischistosomal drug, was administered to 234 healthy adults. It was noted that levels of aflatoxin m1, which is a phase 1 metabolites of AFB1, were reduced by 51% in urine samples of individuals receiving a high dose of oltipraz [58]. Inclusion of vitamins likes A, C and E in the daily diet could also help nullify the toxic effects of aflatoxin exposure to some extent [50]. Chlorophyllin, which is a water-soluble derivative of chlorophyll, is reported to be anti-mutagenic. When chorophyllin and aflatoxin were co-adminitered, this natural product absorbed the toxin before it could reach the liver and reduced AFB1-DNA adduct formation andhepatocarcinogenesis [59,60]. Although the aforementioned preventive measures for lowering aflatoxin levels in foods is necessary but until that becomes a reality, perhaps these drugs or vitamins could be introduced in areas where aflatoxin consumption is unavoidable in order to prevent the development of HCC.

Conflict of Interest

HQ and SSA mined the literature. MI and AAS provided gathered all data related with aflatoxins. NAK and SSH wrote the manuscript. HQ and AAS added and improved the overall manuscriptstructure and contents. All authors read and approved the final manuscript.

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