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The Toxic Effects of Aflatoxin B1: An Update

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Abstract

Aflatoxin B1 (AFB1) is the most toxic in aflatoxin family. It is well known for its involvement in hepatic carcinogenesis. Other adverse effects include immune weakness, reproduction deficiency, malnutrition, and growth impairment. The key mechanism of AFB1 carcinogenesis is supposed to be epoxidation, which produce the AFB1-8,9-epoxide (AFBO) strongly adductive to DNA molecules. Other metabolites like AFM1, AFH1, and AFL, which retain DNA adductive capability, extend its toxicity. Scientists now found that AFB1 also affected epigenetic regulation, which might shed new light into AFB1 toxicity mechanism researches. The detoxification of AFB1 has always been a hot spot in AFB1-related studies. The major methods can be categorized into physical treatment, biological treatment, chemical treatment, combination strategy, and sorbent additives. None of the methods is 100% perfect, however considering economic factors, simplicity, effectiveness, safety, and preservation of the food nutrition. This review will discuss the toxicity and toxic mechanisms of AFB1. Also, detoxification of AFB1 will be reviewed.

Keywords: AFB1, carcinogenesis, DNA adductive capability, epigenetic mutation, detoxification

1. Introduction

Aflatoxins are fungal metabolites majorly produced by *Aspergillus flavus* and *A. parasiticus* [1]. They are recognized as a family of toxic contaminants in a variety of crops, especially promoted by hot and humid climates as well as improper harvesting and storage process [2]. Among these toxins, aflatoxin B1 (AFB1) is the most hazardous, widely known for its “acute intoxication” targeting the liver featured by acute liver failure and death at a short yet high dosage exposure. Other major adverse effects include carcinogenicity and immunosuppression capacity in a chronic way.

2. Toxic effects of AFB1

AFB1 is well known to be hepatotoxic, causing degeneration and necrosis of the liver as well as proliferation of the bile duct and infiltration of inflammatory cells in many species. In recent studies, AFB1 is reported to lead to apoptosis of hepatocytes via an extrinsic mechanism because of high expression of death receptor pathway [3]. Moreover, in 2002, AFB1 has been classified into Group 1 of carcinogens, considering its direct contribution to hepatocellular carcinoma (HCC) [4]. In fact, as early

as in the 1970s, its hepatocarcinogenic property has been testified in animal models [5], and there since, several epidemiological studies from Asia and Africa areas monitored intersection of high HCC incidence and AFB₁ contamination, with 4.6–28.2% of HCC cases globally attributed to AFB₁ exposure [6]. Moreover, hepatitis B virus (HBV) that cooperates with AFB₁ can drastically increase the risk of HCC by 30-fold [7]. Recent researches also find evidences that hepatitis C virus (HCV) also has a synergistic role with AFB₁ in hepatocarcinogenesis. Jeannot et al. found that the incidence of tumorous or pretumorous lesions was elevated by 2.5-fold in AFB₁-treated HCV transgenic mice compared with wild-type mice [8]. Recently, a 20-year clinical follow-up study in Taiwan investigated HCC risk associated with AFB₁ exposure in HCV-positive and HBV-HCV-negative individuals. HCV and AFB₁ exposure were both found as independent risk factors for HCC development. Elevated serum AFB₁-albumin adduct levels were significantly associated with an increased risk of HCC newly developed within 8 years of follow-up in non-HBV-non-HCV participants with habitual alcohol consumption [crude OR (95% CI) for high vs. low/undetectable levels, 4.22 (1.16–15.37)], and HCV-infected participants [3.39 (1.31–8.77)], but not in non-HBV-non-HCV participants without alcohol drinking habit. Therefore, it indicated that AFB₁ exposure contributes to the development of HCC in participants with significant risk factors for cirrhosis including alcohol and HCV infection [9]. What's more, AFB₁ exposure can induce the increased levels of blood glucose in mice and, also, the high probability to develop liver cancer [10].

Liver is not the only target organ of AFB₁ toxification. Its impairment on the immune system has been established in both humans and animals. Several studies linked AFB₁ exposure with reduced levels or functions of immunological factors, such as decreased T or B lymphocyte activity [11, 12], suppressed macrophage or neutrophil effector functions [13], modified synthesis of inflammatory cytokines [14, 15], impaired NK cell-mediated cytotoxicity [16], and thus increasing risk to infectious diseases. A study on Gambian children found that those children were generally exposed to high levels of aflatoxin (as much as 93%). The exposure significantly decreases sIgA in their saliva [17]. In another study, Jiang et al. reported that Ghanaians with higher serum aflatoxin-albumin adducts (AF-ALB) level had lower percentages of CD8⁺ T cells, perforin, and granzyme A than those with the lower level [18]. By comparing HIV-infected individuals exposed to high and low AF-ALB levels with HIV-negative counterparts, experts found that high AF-ALB appeared to accelerate HIV-associated changes in T-cell phenotypes and B cells in HIV-positive participants [19]. This result was in accordance with the observation of Hendrickse et al. long back in 1989 that heroin addicts often experienced rapid progression of HIV infection, while street heroin was often contaminated with aflatoxin and that aflatoxin derivatives were commonly found in the body fluids of the addicts [20]. Recent researches also show that when exposed to a low dose of AFB₁, people were more vulnerable to SIV infection and suffered increased severity [21].

Another chronic adverse effect of AFB₁ is malnutrition and growth impairment. Several observations in developing countries have pointed out the accordance of kwashiorkor occurrence with high AFB₁ concentration in sera [22–24]. Animal models and in vitro methods have also been used to investigate the mechanism of AFB₁-induced malnutrition. AFB₁ is supposed to alter metal absorption and bioavailability. Long back in the 1990s, broiler chicks exposed to AFB₁ were found to have lowered serum concentrations of vitamin D and calcium possibly due to impairing renal function and altering parathyroid metabolism [25]. AFB₁ had toxicity toward the vitamin D receptors, interfering with the actions of vitamin D on calcium-binding gene expression in the kidney and intestine, which increase the risk of rickets. Experimental data indicated a 58 and 86% decrease if the cells were exposed to 5 and 50 ng/mL of AFB₁, respectively [26]. Dietary intake of AFB₁ also leads to vitamin A

depletion in young broiler chicks, which could be attenuated by additional feeding of NovaSil PLUS, an AFB1-binding agent [27]. Other adverse effects included inactivating enzymes in liver lipid metabolism [28] and redistribution of various metal ions in the central nervous system and genital glands [29]. Mechanistically, it is unclear how AFB1 alters nutrient levels. Existing hypothesis includes impairment of liver, absorption deficiency, and insulin-like growth factor modulation. Ubagai et al. found that AFB1 could modulate the insulin-like growth factor (IGF)-2 signaling axis [30]. Furthermore, Castelino et al. found that Kenyan schoolchildren that exhibited high levels of AFB1-albumin were shorter and had lower levels of IGF1 and IGFBP3 than groups with lower levels of the AFB1 biomarker [31]. Additionally, AFB1 may affect nutrient absorption through intestinal toxicity. Enteropathy features decreases in intestinal density and increases in fibrosis/necrosis, which has been shown in poultry experimental models [32]. The disruption of intestinal function by AFB1 is due to the destruction of epithelial cells and their organization due to the inhibition of protein synthesis or cytotoxicity due to DNA and protein binding. This tissue damage can alter the structure of intestinal epithelial cells, affecting the efficiency of nutrient absorption [33, 34]. Recent studies also found that lack of protein [35] and vitamins A, C, and E [36] together with aflatoxicosis worsened hepatic oxidative stress. These findings gave rise to the theory that toxification and malnutrition can form a vicious circle, which add up to the damage of AFB1.

AFB1 is not likely to be limited by the physical barrier. Aflatoxin metabolites and biomarkers can be tested in breastmilk and cord blood, which means this toxin can affect fetuses and neonates. Levels of aflatoxins in pregnant women have also been linked to poor birth outcomes in humans. An early Kenyan study indicated that newborns birthed from aflatoxin-positive mothers had significantly lower birth weights. The relation between AFB1 maternal exposure and growth impairment was significant and may even lead to stillbirth [37]. Another study found a strong negative correlation between aflatoxin levels and birth weights in Gambian infants [38]. Also, Shuaib et al. reported that mothers in the highest quartile of serum AFB1-albumin levels were highly likely to have lower birthrates than mothers with lower concentrations [39].

AFB1 not only interferes with the reproductive process after embryo forms. The findings of the current study repeatedly demonstrate direct effects of dietary toxin intaking on spermatozoa, oocytes, and in vitro fertilization while exploring the potential hazards associated with AFB1 exposure [40]. Low dose of AFB1 exposure was reported to cause follicular atresia in ovaries in animal models [41]. AFB1 was also testified to impair sperm quality and fertilization competence in animal and in vitro models, although the mechanism underlying cellular damage has not been fully elucidated. In summary the alterations include impairment of (1) membrane integrity, (2) mitochondrial function, (3) DNA integrity, and (4) fertilization competence. Exposure of fresh semen to 10 μ M AFB1 prefertilization resulted in sperm DNA damage and impaired fertilization competence, featured by reduced proportion of oocytes that are divided to early-stage embryos after fertilization [42]. Exposure of sperm to low concentrations of AFB1 for a few hours also resulted in decreased sperm viability and hyperpolarization of the mitochondrial membrane, which was most remarkable in ejaculated sperm, suggesting that later stages of spermatogenesis were affected. AFB1 may adversely affect the spermatogenesis through mitochondria-dependent apoptosis via inducing oxidative stress, diminishing cellular mitochondrial content, and enhancing pro-apoptotic Bax, caspase-3, and p53 expression [43].

Besides all mentioned above, AFB1 also induces damage to the heart [44–46], kidney [45], and even central nervous systems by direct toxicity to both the neural cells and blood-brain barrier [47–49]. It is of great importance to better understand how this toxin functions in order to discriminate its threats to public health.

3. The metabolism and toxic mechanism of AFB1

It is well established that AFB1 epoxidation is the key step in the genotoxic process leading to carcinogenesis. The predominant place of epoxidation is in the liver by the P450 family before transforming into the final carcinogen aflatoxin B1-8,9-epoxide (AFBO), which has two isomers: endo-8,9-epoxide and exo-8,9-epoxide [50]. CYP3A4 is the major producer of AFBO formation at high AFB1 concentrations, producing primarily the exo isomer of AFBO [51], while lower concentration of AFB1 exposure makes CYP1A2 the main producer of AFBO in turn. Additionally, CYP1A2 was found to produce more of the exo isomer than CYP3A4 at these low concentrations [52, 53]. The highly electrophilic nature of AFBO allows it to form adducts to amines in proteins or nucleic acids and then interrupt the function of these biological molecules. Since the exo isomer has a much higher affinity for guanine residues than the endo isomer, AFB1-exo-8,9 epoxide, which has the highest concentration in the liver, is therefore considered to be the major carcinogenic metabolite [54, 55].

AFB1 is also metabolized into a number of hydroxylation products through the P450 system. These include aflatoxin Q1 (AFQ1), aflatoxin P1 (AFP1), aflatoxin B2a (AFB2a), aflatoxin M1 (AFM1), aflatoxicol (AFL), and aflatoxicol H1 (AFH1), and among them, AFQ1, AFP1, and AFB2a were traditionally considered detoxification products of AFB1 due to their much lower DNA-binding potential [56–58]. In the recent decades, AFQ1 was found to have a potential as a biomarker for AFB1 exposure indication. AFQ1 consists of 1–11% of the AFB1 hydroxylation products indicating that in humans, AFQ1 production occurs frequently and at high enough levels to detect [59]. In 2005, a study evaluated fecal and urinary excretion of AFM1, AFQ1, and AFB1-N7-guanine in 83 Chinese males. The study demonstrated that AFQ1 is excreted in urine and feces at higher levels than AFM1, especially in feces, since fecal samples contained approximately 60 times more AFQ1 than AFM1. Additionally, fecal concentrations of AFM1 and AFQ1 were higher than in urine, indicating the usefulness of feces as a predictive marker for AFB1 exposure [60]. However, as the most prominent metabolite of AFB1, AFB2a has an interesting property as compared to many of the other metabolites which has the ability to bind to cellular proteins through the formation of a pyrrole ring. Additionally, this binding only occurs on primary amines that is favored in alkaline pHs. This adduction can also occur on phosphoethanolamine head groups on phospholipids, forming a unique structurally characterized aflatoxin-lipid adduct [61]. The protein-binding capability of AFB2a is thought to contribute to other potential cellular toxicities. For example, AFB2a binding has been shown to inhibit the activity of deoxyribonucleases, demonstrating the ability of AFB2a to alter enzymatic activities of target proteins [62]. Chen et al. recently observed mitochondria-mediated apoptosis and PI3K/Akt/mTOR-mediated autophagy induced by AFB2 in hepatocytes of broilers [63]. AFB2a has been found in high levels in poultry eggs possibly as a result of avian metabolism, providing a potential source of dietary exposure [64]. AFM1 is a major metabolite produced by CYP1A2 and is the most carcinogenic of the hydroxylated metabolites. This is supported by the DNA-binding effect of AFM1 which has been demonstrated in rat, mouse, and pig and has even been identified to form an N7-guanine adduct similar to AFB1 [65]. Just like AFB1, AFM1 can be excreted in cattle or human milk [66]. AFM1 is also excreted in high levels in urine following AFB1 exposure and thus has become an additional biomarker of AFB1 exposure [67]. AFL is found in the cytosolic fractions of liver preparations and is formed by an NADPH reductase, usually in the cytosol [68, 69]. AFL retains its DNA-binding activity and has been shown to be enzymatically reconverted back into AFB1 acting as a reservoir for AFB1, extending its toxic effects [69]. Despite excretion in breast milk and urine, AFL is the sole metabolite that is able to be transferred through the human placenta, and,

furthermore, AFL is the only metabolite that is formed from AFB1 by the placenta itself. This indicates that AFL may play a large role in developmental toxicities of AFB1 [69]. AFH1 has a structure similar to AFL which contains an additional hydroxy group on the terminal cyclopentenone ring, which implies a similar toxicity. The metabolic formation of AFH1 was found to be dependent on two enzyme systems: the microsomal hydroxylase and cytoplasmic reductase systems [70].

By firmly binding to purine bases (usually at the N7 position on guanine) of DNA, epoxide intermediate forms AFB1-N7-guanine (AFB1-N7-Gua) adduct, and thus promoting mutations in nucleotide sequence. The charged adduct causes depurination and thus apurinic site formation [71]. The predominant mutation caused by AFB1-N7-Gua adduct has been identified to be the G → T transversion on the site of the original adduct [72]. Moreover, the mutation has a favor for specific base pair locations. Mace et al. reported a selective affinity for guanine bases with a guanine or a cytosine as 50 bases and more specifically at the third base of codon 249 of the p53 tumor suppressor gene [73]. This mutation was found really common in a great number of epidemiological studies on HCC patients from regions of high aflatoxin exposure, strengthening the association between HCC incidence and aflatoxin exposure [74].

Interestingly, AFB1 can be activated by other biotransformation aside from the principal pathway involving CYP. As early as in 1985, Battista et al. have described the epoxidation catalyzed by prostaglandin H (PGH) synthase [75], whereas Weng et al. hypothesized that lipid peroxidase (LPO) was one of the mechanisms behind AFB1 carcinogenesis, triggering the production of cyclic α -methyl- γ -hydroxy-1,N2-propeno-dG (meth-OH-PdG) adduct and inhibiting DNA repair [76]. Another important role in DNA damage and thus carcinogenesis is played by oxidative stress. A recent study showed that the AFB1 treatment resulted in a significant and concentration-dependent increase in intracellular ROS production, whereas mitochondrial functions such as glutamate/malate and succinate-driven respiration were significantly uncoupled. Those oxidative stress effects activated mitochondrial ROS-dependent signal pathways, which induced apoptosis through the mitochondrial signal pathway [77]. What's more, there are some proteins modulated by AFB1 like CYP1A2, CYP1A5, CYP3A4, TP53, GSMT1, MDM2, CAT, OGG1, IRS1, IRS2, SRC, AKT1, MAPK1, MAPK3, and PDK1. These proteins are involved in important metabolic pathways such as FoxO signaling pathway, PI3K-Akt signaling pathway, AMPK signaling pathway, MAPK signaling pathway, and VEGF signaling pathway [65]. In fact, those modulations induce both tumor progression and chemotherapy resistance as well as in other pathogenetic procedures.

To further investigate the mechanism behind nuclei damage, epigenetic changes cannot be omitted. Accumulating evidence also indicates that epigenetic modifications play a vital role in AFB1-induced carcinogenesis and more attention is being paid to them [78]. It is believed that epigenetic aberrations play important roles in tumorigenesis, pathways, and development of carcinogenesis including tumor invasion, initiation, and plasticity [79, 80]. Those alterations include oncogene activation, tumor suppressor gene inactivation, and chromosomal instability (CIN), which then interfere with the critical signaling pathways. Epigenetic alterations include three kinds of modifications, DNA methylation, histone modifications, and regulation of noncoding RNA (specifically small noncoding RNAs, microRNAs) [81, 82].

DNA methylation, which regulates the gene expression level, is one of the frequent epigenetic events in cancer development. The formation of AFB1 adducts is believed to cause methylation alteration of some important genes regulating the mutagenesis of AFB1 in the carcinogenetic development of HCC. In *in vitro* studies, Rieswijk investigated the persistent AFB1-induced impact on the DNA methylation footprint in relation to the transcriptome in HCC for the first time. By comparing the transcription level and DNA methylation degree, a number

of persistent hypomethylated and upregulated genes named PCNA, RAB27A, TXNRD1, DIAPH3, HIST1H2BF, and CCNK were identified to have an alteration on the transcriptome level. PCNA acts as a scaffold to recruit proteins involved in DNA replication and DNA repair including mismatch repair, cell cycle, apoptosis, chromatin remodeling, and preservation of epigenetic marks [83, 84]. RAB27A expression was closely correlated with tumor progression [85]. Upregulation of TXNRD1 decreases the expression of AFB1 aldehyde reductase (AFAR) and glutathione-S-transferase (GST) that are responsible for the detoxification of AFB1-8,9-epoxide via glutathione conjugation into less active metabolites, thereby promoting the formation of AFB1 adducts with macromolecules in the liver [86]. CCNK can bind with CDK12 to form the complex CCNK/CDK12 and regulate the expression of several DNA damage response genes and some critical regulators of genomic stability [87]. Moreover, Zhang et al. conducted a number of human studies about AFB1-induced methylation alterations in HCC. By investigating promoter methylation status in tumor suppressor and other cancer-related genes such as p16, RASSF1A [88], O6-methylguanine-DNA methyltransferase (MGMT) [89], glutathione-S-transferase p1 (GSTP1) [90], and the level of AFB1-DNA adducts in HCC tissue samples from Taiwan, they found that frequent aberrant methylation in the CpG islands of promoters of the genes mentioned above is closely related to hepatocarcinogenesis. In addition, the correlation between AFB1-DNA adducts and hypermethylation of these genes was found to be statistically significant. Since AFB1 plays an important role in the development of HCC, it can be speculated that AFB1 may bind preferentially to methylated CpG sites or specific structures in chromatin, inducing damage to DNA that may impact methylation [91]. Furthermore, according to the analysis of progression from the normal liver to HCC proposed by Herath et al. [92], under the aflatoxin exposure, methylation appears to precede cirrhosis in a subset of tumors, while tumors appear to be able to progress in the absence of cirrhosis, which may be a consequence of hypomethylation. The association between AFB1-DNA adducts and RASSF1A methylation in HCC was statistically significant. Multivariate analyses including the mRNA, protein expression, and methylation status of RASSF1A in HCC showed that RASSF1A hypermethylation was related to AFB1-DNA adducts. Besides, there seemed to be little effect of RASSF1A methylation on patient survival [93]. In whole-animal studies, Baik investigated the DNA methylation status of the rat gamma-glutamyl transpeptidase (GGT) gene in embryonic, adult, and neoplastic liver using rats treated with AFB1-contaminated peanuts in 1991. The methylation patterns of the GGT gene were investigated in AFB1-induced HCC tumors, and hypermethylation was observed. However, the regulatory mechanism of GGT methylation after AFB1 exposure was not clear [94]. In addition, it was observed to be more than partial hypermethylation in the analyzed CpG sites of the p19Arf promoter, and methylation of transcription factor-binding sites or consensus sequences was confirmed in lung tumors of mice [95]. Wu assessed the relationship between AFB1 exposure and global DNA methylation. The methylation status of DNA in white blood cells from cancer-free participants was investigated through pyrosequencing, and the results showed that decreased Sat2 or LINE-1 methylation was associated with dietary exposure to AFB1, supporting the hypothesis that AFB1 might play an important role in HCC by altering the global DNA methylation status and that exposure to AFB1 induces global DNA hypomethylation [96].

AFB1 exposure and DNA methylation are reported to be associated with embryonic development as well. In vivo, when pregnant women are exposed to AFB1, the DNA methylation status of their infants may be constantly influenced until 2–8 years old. Exposure to AFB1 gives rise to methylation alterations of some important genes including growth factor genes, such as IGF1 and FGF12, and

immune-related genes, TGFBI, TLR2, and CCL28 [97]. In vitro porcine models and oocyte exposure to AFB1 (50 μ M) were revealed to appear increasing DNA methylation levels and oxidative stress, which may explain the excessive autophagy and apoptosis [98].

Histone, known as the chromatin-related protein, plays an important role in posttranslational modification. So, it is reasonable to speculate that AFB1-induced epigenetic mutation in histone is another mechanism of carcinogenesis. As early as in 1980, Groopman and Wogan used radiolabeled [3H] to mark the in vivo footprint of AFB1 residues in the rat liver. They observed that after 36 h of feeding marked AFB1, approximately 5–10% of the total nuclear-bound aflatoxin residues distributed in histones. Among all the histones, histone H1 was the major protein target for nuclear AFB1 binding, with an adduct level of three to four times higher than other companions [99]. Studies focusing on AFB1-induced reproductive toxicity also had a positive finding in histone. When investigating the relationship between AFB1-induced epigenetic modifications and mammalian oocyte maturation, in 2015, Liu observed toxic epigenetic modifications in porcine oocyte of exposure to 50 μ M AFB1. Results indicated that the levels of transcription marks H3K27me3 and H3K4me2 decreased, while the level of H3K9me3 increased [98]. H3K27me3 is responsible for silencing the expression of key developmental genes during embryonic stem cell differentiation [100]. H3K4me2/H3K4me3 and H3K9me3 are supposed to play an important role in the efficient reprogramming of pluripotency genes in somatic nuclei and thus determine cell fate [101, 102]. Another similar research study on mice gave up to the similar conclusion. Mice were fed with mycotoxin-contaminated maize and observed for histone change in their oocytes. According to the fluorescence intensity analysis, the levels of H4K20me3 and H3K9me3 increased, while the levels of H4K20me2 and H3K27me3 decreased [103]. The results indicated that mycotoxins including AFB1 may improve the transcriptional activity of the oocyte genome by reducing the H3K27me3 levels and may affect the chromatin configuration and cell cycle progression in oocytes via altering the H3K9me3 levels, reducing H4K20me2 levels, and increasing H4K20me3 levels. In addition, the DNA methylation level of oocyte in these two findings was both found to be increased. Ghufran et al. investigated AFB1-induced effects on an epigenetic regulatory protein, arginine methyltransferase 5 (PRMT5), in human cell lines for the first time in 2016 [104]. Upregulation of PRMT5 was observed after AFB1 treatment, which might play an important role in AFB1-induced tumorigenesis. However, the mechanism of upregulation of PRMT5 in AFB1-induced cancer remains obscure. Furthermore, besides histone methylation, AFB1 was also found to affect histone acetylation and deacetylation in the liver. Results showed that AFB1 exposure increases the rate of deacetylation of histone fractions F2A1 and F3 [105].

MicroRNA (miRNA) is a kind of short-chain noncoding RNA which plays an important role in gene expression modulation. The previous research mainly focused on the regulation of miRNAs in AFB1-induced disease and tumorigenesis. In a study detecting the level of AFB1-DNA adducts and the expression of miRNA-429 [106] and miRNA-24 [107] in tumor tissues, miR-429 and miR-24 were found to be upregulated in HCC tumor tissues with high AFB1 exposure, and their high expression was significantly correlated with larger tumor size. Importantly, overexpression of these two miRNAs inhibits apoptosis, induces progression of tumor cell growth, and had a positive correlation with the levels of AFB1-DNA adducts, indicating the potency as biomarkers for AFB1-related HCC prognosis and tumorigenesis of those two miRNAs [106, 107]. Further, polymorphisms in pre-miRNAs were investigated for the potential as risk factors or prognostic biomarkers of AFB1-induced HCC in a large case-control hospital research in China, in which rs28599926 in miR-1268a was identified as one candidate [108]. Later,

several studies about the mechanisms underlying the signaling pathway involved in these processes were conducted. Liu et al. found that in vivo upregulation of rno-miR-34a-5p leads to cell cycle arrest by downregulating the expression of the cell cycle-related genes MET, CCNE2, and CCND1. It also facilitated p53 repair of DNA damage in the liver of AFB₁-treated rats. Thus, miR-34a-5p might be considered as a sensitive biomarker of AFB₁-induced DNA damage in the liver [107]. Zeng hypothesized that the dysfunction of a novel GSK-3b-C/EBPa-miR-122-IGF-1R regulatory circuitry was the mechanism behind the development of HCC [109]. In vitro studies on different expression profiles of miRNAs identified several cancer-related microRNAs and the predicted target genes involved in cancer-related pathways. Zhu et al. found that upregulation of miR-34a might suppress the Wnt/ β -catenin signaling pathway in HepG2 cells anticipated by AFB₁ [110]. Another functional studies revealed that miR-138-1* inhibited proliferation, colony formation, migration, and invasion of P50 B-2A13 cells and might affect AFB₁-induced malignant transformation through targeting PDK1 [111].

The majority of these studies investigated the regulation of miRNAs. One study, however, reported the changes of lncRNA expression in AFB₁-induced HCC formation. The upregulation of H19 gene encoding lncRNA was found to promote cell growth and invasion in human HepG2 cells after AFB₁ treatment [112]. Since lncRNA is a novel hot spot of noncoding RNA researches, the information is limited. More detailed studies are required to establish the signaling pathway involved in this process to understand the mechanism.

4. Detoxification strategies

It is reported that carry-over of AFB₁ as AFM₁ in the milk of dairy cows has been established to range from 0.3 to 6.2% [113]. In consideration of this high prevalence of AFB₁ in food, it is of great priority to find effective strategies to prevent or remove contamination in order to restore the safety and edibility of food products. Prevention of *Aspergillus* infection can stop the contamination from the source. The control strategies include the use of genetically altered crops that are resistant to *Aspergillus* and environmental stressors, pesticide usage, crop rotation, and timing of planting, together with proper drying, packaging, storage, and preservative/pesticide usage to suppress fungal reproduction. These strategies however are not enough to fully inhibit contamination, so further postharvest techniques are being developed to detoxify contaminated foods. These then involve the knowledge of physical and chemical characteristics of AFB₁. The characteristic frame of AFB₁ is the fusion of a cyclopentenone ring to the lactone ring of the coumarin structure. The toxin favors polar organic solvents and is barely soluble in nonpolar solvents and slightly soluble in water. AFB₁ remains stable even at high temperature (>100°C), so that regular thermal procession cannot detoxicate it during food manufacturing. This thermal stability makes a great obstacle in the reduction of aflatoxin in milk and dairy products, since the wild used sterilization such as pasteurization, etc. all belonging to thermal treatment. Other physical or chemical properties led to the development of other methods of decontamination in order to assure a good level of decontamination without disruption of the nutritional properties or safety of feed. Those strategies can be classified into physical treatment, biological treatment, chemical treatment, combination strategy, and sorbent additives.

Physical means of removing AFB₁ from foods are most commonly heating comparatively to a higher temperature or for a longer time and irradiation using gamma (γ) rays. AFB₁ is reported almost completely degraded at harsh heating temperatures as high as 160°C and above [114]. Prolonging of heating time and

additional humidity can help the detoxification [115]. However, such a strategy may disrupt the integrity of product after heating/roasting is complete. This advantage inhibits the popularity of the maximum temperature that can be used, resulting in incomplete removal of AFB₁. The other most commonly reported physical decontamination method is γ radiation. This technique has been widely tested in a variety of food substrates, with an average percent reduction of 65%. The fundamental of γ radiation is production of ionizing radiation. According to a recent study, the effective concentration ranged from 6 to 60 kGy [115].

Biological-based interventions have also been investigated for their potential in reducing AFB₁ levels. Lact microbes such as *Lactobacillus*, *Saccharomyces*, *Cellulosimicrobium*, and others have been identified to enzymatically convert aflatoxin, zearalenone, ochratoxin, patulin, fumonisin, deoxynivalenol, and T-2 toxin to less toxic products [116]. The reduction averaged approximately 86% across recent studies. Despite microbe degradation, herbal extract incubation is another biological strategy. Dissolving AFB₁ in aqueous extracts of various plant species is also highly effective. For example, according to the reported data, extracts from *Adhatoda vasica* Ness and *Corymbia citriodora* both achieved >95% degradation of AFB₁ [117]. Treatment time using those two approaches however is very long, usually requiring several days to carry out [118]. It would allow an increasing efficiency of those processes by further identification of the active components responsible for the degradation. Therefore, the use of purified enzymes from various biological sources has been investigated for AFB₁ degradation potential. Those agents have included laccases, manganese peroxidase, and the recently identified *Bacillus* aflatoxin-degrading enzyme. The efficacy of these approaches has been high, but more information based on further practice on real food substrates is necessary. As is the case with all the biological control methods, the time of treatment is still long, taking several days to complete which may not be feasible in large-scale applications [119–121]. Another disadvantage lies in an uncertainty of the end products of the treatments, which makes the safety of treated foods hard to determine.

Chemical additives are also widely used to detoxify contaminated foods in the food industry. Acidification, including citric, lactic, tartaric, and hydrochloric acid, has shown high efficiency in reducing toxicity of AFB₁-contaminated foods. These methods have been observed to have high reduction rate in less than 24 h even at room temperature. The conducting process is also simple, just requiring to soak contaminated foods in acidic solutions for a given amount of time [61, 122, 123]. Unlike biological degradation, the detoxification product of AFB₁ in acid is AFB₂a as mentioned above, which is widely recognized as a far less toxic metabolite of AFB₁. On the other hand, ammoniation which refers to breaking down AFB₁ in an alkaline environment is also effective in detoxification. When foods are treated with either gaseous or liquid ammonia (usually 1.5–2%), the degradation rate can sometimes reach above 99% in comparatively long time, ranging from 24 h to 15 days [124, 125]. The degradation product due to ammoniation is a less mutagenic metabolite of AFB₁, known as aflatoxin D₁ (AFD₁), formed through hydrolysis and decarboxylation, though risking a reversion back into AFB₁ if reset in an acidified environment [126]. Finally, ozonation is another commonly used chemical control method. Ozonolysis at a concentration of 6–90 mg/L has been shown to quickly degrade AFB₁ by 86.75% in as little as 20 min in wheat. Other recent studies have treatment time range from 30 min to 96 h, mostly under 180 min, and all have seen >65% reductions. Additionally, ozone has been investigated in a variety of foodstuffs, indicating a stable effectiveness and reliable safety [115, 127, 128]. According to an analysis of the breakdown products of AFB₁ after ozonolysis by Diao et al., the moieties responsible for mutagenicity disappeared, indicating that these products are likely less toxic, although it has not been verified using mutagenicity assays [115].

All those methods mentioned above solely have had moderate to high success in reducing AFB1 levels; nevertheless, the highest efficacies are seen when they are combined with one another. The combination of ammonia and heat has been shown to drastically reduce treatment time from several days to 15–120 min and repeatedly manifested >99% reduction of AFB1 in a series of studies [129–131]. Proctor et al. reported that heating along with ozonation turned out to have reduced treatment time and lower effective temperature while remaining as high as 77% reduction of AFB1 in peanuts at 75°C after only 10 min [132]. Treatment of contaminated foods with alkaline substances (other than ammonia) along with heat is another effective method. Nixtamalization, the tortilla-making process employing heat and alkaline calcium hydroxide, has been shown to decrease AFB1 by approximately 84 and 90% in two separate studies [133, 134]. The applications of high temperatures (80–120°C) together with acidification such as HCl, citric acid, and lactic acid were also reported to have high degrading potential (85–100%). The addition of citric acid can help degrade 98% of AFB1 at 100°C in 20 min [135–137]. The combination of heat and acidification is attractive with the fact that those two methods separately have already been widely used in food manufacturing industry, so that no specialized or expensive equipment is needed additionally. A unique combination has recently been performed by Rushing and Selim where acidification has been used with heat and arginine. This causes AFB1 to form a stable pyrrole ring with the amino acid, which is completely non-genotoxic and inabsorbable across the intestinal tract. This method was able to completely converse AFB1-contaminated corn in 20 min. This result advocates for adding amino acids to the previous acidification treatments to form stable, nontoxic forms of AFB1 [137].

Another unique approach to solving AFB1 contamination is the addition of sorbents to food. This method is different from the degradation methods because it prevents the hepatotoxic effects of AFB1 by acting as binding agents to prevent absorption of AFB1 across the intestinal tract after ingestion rather than destroying or reducing the amount of AFB1 in the food as mentioned above. Chlorophyllin and chlorophyll as the most well-studied of those agents were observed to reduce AFB1-DNA adduct by 37% in rainbow trout and in turn reduce tumor incidence by 77%, when added to the contaminated feed [138]. Simonich et al. reported that when AFB1-contaminated feed was added with chlorophyllin and chlorophyll, AFB1-DNA adducts, AFB1-albumin, and urinary AFM1 levels in rat reduced by 42, 65, and 90% compared with 55, 51, and 92%, respectively. Accordingly, the tumor incidence in these rats was reduced by 74 and 77%, respectively [139]. Egner et al. found that, by introducing chlorophyllin into the diets of humans in high-risk areas for AFB1 exposure, AFB1-N7-guanine levels in those subjects reduced by 55% compared to that in individuals who were not fed with this agent [140]. In another clinical study, four volunteers were given a single dose of 30 ng of AFB1 with either the co-administration of chlorophyll and chlorophyllin or not. Results showed that chlorophyllin and chlorophyll reduced urinary AFM1 levels by 28 and 41%, respectively [140]. Those data above show that sorbents or binding agents in diets in high-risk areas can partially alleviate the toxic effects of AFB1.

Clay is another commonly studied enterosorbent. With a mechanism similar to that of chlorophyllin and chlorophyll, clays have been shown to protect against AFB1 toxicities in multiple animal models by reducing AFB1 absorption and reducing AFM1 levels in milk. NovaSil (a calcium montmorillonite clay) is particularly successful in reducing the toxic effects of aflatoxin-contaminated feed and AFB1 biomarkers in humans [141]. In long-term animal study, no overt toxicities were observed when rats were fed up to 2.0% of NovaSil in their diet for 28 weeks, indicating the safety of using this substance [142]. In a clinical trial, Ghanaians in high risk for AFB1 exposure were given an oral bolus of placebo, low dose, or

high dose of NovaSil. Participants in the low and high dose had significantly lower AFB1 biomarkers such as urinary AFM1 and serum AFB1-albumin after a period of 3 months. Additionally, during the observation, only mild, infrequent adverse effects were reported (such as nausea, diarrhea, heartburn, and dizziness), and no significant side effects were monitored by evaluating liver, kidney, or hematological parameters of all the individuals [143, 144]. These results indicate that the addition of NovaSil into the diet can be a safe and effective method to reduce AFB1 toxicity.

5. Future direction and conclusions

In the past decade, several studies have emphasized the important role of AFB1 in public dietary health. The high frequency and levels of AFB1 recently found in food supplies of various countries, particularly in Africa and Asia, indicate that exposure of populations to this toxin still remains largely uncontrolled. The toxin is then well known for its strong carcinogenesis potential. In fact, when further investigations were made, AFB1 was found to cause function feebleness of nearly all organs and systems. Furthermore, AFB1 has become an occupational hazard for those working in the food industry, leading to some particularly high rates of exposure. Evidence that has been gathered over the last several decades has shown the clear carcinogenic effect of AFB1. Also, the negative effect of AFB1 on nutritive status, growth/development, and immune system function is becoming clearer.

So as to disrupt or reverse AFB1-related pathobiological process, it is necessary to have a better understanding of the mechanism of AFB1 toxicity. Instead of stating AFB1 impairment to individual organ, a further exploration into the genotoxicity and epigenetic toxicity should be more proper, which has become a future trend in novel toxicity testing. However, researches focus on the relationship between histone modifications and AFB1 exposure, and the regulation of other noncoding RNAs except for miRNAs in AFB1 toxic mechanisms is rare. More effort is needed in related research. The network between epigenetic and genetic mechanisms in AFB1 toxicity needs further exploration, since genetic changes and epigenetic changes influence each other in most of the pathobiological process [92]. In addition, in the past few years, a variety of single-cell technologies have shed light on the extraordinary variability and accuracy of AFB1 toxicity. These technologies provide more opportunities to study the mechanism of AFB1 toxicity at the single-cell level, which is the central theme of recently raised concept precision toxicology [145].

In conclusion, AFB1 contamination remains an unneglectable threat to public health in developing countries. Its effect on malformation and other health problems may have been underestimated due to data deficiency. Despite the effectiveness of existing detoxification methods mentioned above, those methods are far from being popularized. Perhaps over time, these methods will see increased usage in order to provide cleaner foods worldwide.

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