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Microcystin-LR increases genotoxicity induced by aflatoxin B1 through oxidative stress and DNA base excision repair genes in human hepatic cell lines☆

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ABSTRACT

Aflatoxin B1 (AFB1) and microcystin-LR (MC-LR) simultaneously exist in polluted food and water in humid and warm areas, and each has been reported to be genotoxic to liver and associated with hepatocellular carcinoma (HCC). However, the genotoxic effects of the two biotoxins in combination and potential mechanism remain unknown. We treated the human hepatic cell line HL7702 with AFB1 and MC-LR together at different ratios, examined their genotoxic effects using micronuclei and comet assays, and evaluated the possible mechanism by measuring oxidative stress markers and DNA base excision repair (BER) genes. Our data show that co-exposure to AFB1 and MC-LR significantly increased DNA damage compared with AFB1 or MC-LR alone as measured by the levels of both micronuclei and tail DNA. Meanwhile, AFB1 and MC-LR co-exposure showed biphasic effects on ROS production, and a gradual trend towards increased Glutathione (GSH) levels and activity of Catalase (CAT) and Superoxide Dismutase (SOD). Furthermore, MC-LR, with or without AFB1, significantly down-regulated the expression of the base excision repair (BER) genes 8-oxoguanine glycosylase-1 (OGG1) and X-ray repair cross complementing group 1 (XRCC1). AFB1 and MC-LR in combination upregulated the expression of the BER gene apurinic/apyrimidinic endonuclease 1 (APE1), whereas either agent alone had no effect. In conclusion, our studies show that MC-LR exacerbates AFB1-induced genotoxicity and we report for the first time that this occurs through effects on oxidative stress and the deregulation of DNA base excision repair genes.

1. Introduction

Food and water provide humans with their basic nutritional requirements, but are sometimes unavoidably contaminated by environmental toxins. Such environmental contaminants may have combinative effects on health even at low-level exposure. Traditional food and water treatment methods, such as washing the food and filtering the water, may not completely remove all contaminants. Moreover, in developing countries the situation may be worse due to reduced regulations and poorer infrastructure. In spite of the fact that humans are commonly co-exposed to environmental contaminants, existing studies on their effects in combination are still uncommon (Noyes et al., 2009).

Hepatocellular carcinoma (HCC) is one of the most serious cancers worldwide, with 500,000–1,000,000 newly-diagnosed cases (Gomaa et al., 2008) and 600,000 deaths (Kucukcakan and Hayrulai-Musliu, 2015) annually. Risk factors for HCC are complex, including hepatitis virus infection, heredity, lifestyle, and environmental exposure to toxins. Indeed, environmental toxins make a major contribution to liver carcinogenesis, especially in the combined exposure participants, but are unfortunately often overlooked (Yu and Yuan, 2004).

Aflatoxins (AFTs) and microcystins (MCs), commonly found in polluted food and water in humid and warm areas, have been reported to be genotoxic to the liver by many *in vitro* and *in vivo*

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studies. Aflatoxin B1 (AFB1) has been proven to be an important hepatocarcinogen, with 4.6–28.2% of HCC cases globally attributed to AFB1 exposure (Kucukcakan and Hayrulai-Musliu, 2015). Moreover, HBV can further elevate risk of HCC in AFB1-exposed people by 30-fold (Liu and Wu, 2010). Aflatoxin B1 (AFB1) is the most potent teratogen of all AFTs, and is classified as a high level potential carcinogen by the International Agency for Research on Cancer (IARC). Some studies have certified AFB1 to be hepatotoxic because it can induce liver damage in mammalian species (Choi et al., 2011; Kim et al., 2011), fish (Arana et al., 2014) and poultry (Yang et al., 2012; Liao et al., 2014) under acute exposure, and other studies have also shown that AFB1 can cause hepatocellular carcinoma (HCC) through complex pathways after chronic exposure (Rapisarda et al., 2016). Meanwhile, Microcystin-LR (MC-LR) has been confirmed to be carcinogenic through inducing DNA damage and gene mutations, and through interfering with DNA repair, although no human studies have reported its direct association with HCC (Zegura, 2016). MC-LR is the most potent teratogen of all MCs, and is classified as a type II B potential carcinogen by IARC. MC-LR exposure has been shown to cause acute damage to liver cells *in vitro* (Mereish and Solow, 1990; Ding et al., 2000) and also in animal studies (Weng et al., 2007; Lezcano et al., 2012) and is a proven carcinogen due to its ability to induce oxidative stress and inhibit protein phosphatases (including PP1 and PP2A) (Svircev et al., 2010).

AFTs, metabolites of *Aspergillus flavus, A. parasiticus, A. tamari* and *A. nominus* (Goto et al., 1996), can easily contaminate crops consumed by humans and animals, with five billion people being chronically exposed worldwide (reported in 2006) (Strosnider et al., 2006). AFTs are widespread in Sub-Saharan Africa, Eastern Asia, and parts of South America (Kew, 2013), usually endogenous to the regions located between 40° north and 40° south. MCs are metabolites of blue-green algae, and can contaminate water and aquatic food in the same areas threatened by AFTs (Zurawell et al., 2005). Our previous study found that residents in Southwest China were commonly exposed to both AFTs and MCs through intake of contaminated food and water, and the estimated daily intakes (EDIs) of AFTs and MCs were 8.31 and 4.05 ng per day per kilogram, respectively (Lin et al., 2016). AFTs and MCs are both confirmed to accumulate in their primary target organ, the liver (Wang and Groopman, 1999; Robinson et al., 1989).

The carcinogenic effects of the combination of AFB1 and MC-LR have been demonstrated in animal experiments. A study conducted in rats published in 1999 found that co-exposure to MC-LR and AFB1 may significantly promote hepatocarcinoma (Sekijima et al., 1999); Min Lian et al. also found that co-exposure to AFB1 and MC-LR in mice tends to elevate the incidence of liver tumors compared with exposure to single agents (Lian et al., 2006). Hashimoto et al. reported that AFB1 and MC had a synergic mutagenic response through inducing higher rate of micronucleus than the single agent in *Tilapia* (Hashimoto et al., 2012). Our previous study conducted in the Three Gorges Region have shown that MC-LR increases the liver injury risk induced by combined exposure to AFB1 and HBV, although the combined effect on HCC have not been found yet (Liu et al., 2017). In the study period, we also found that MC-LR, rather than AFB1, might be one important risk of renal-function impairment and the co-effects of MC-LR and AFB1 on the renal-function was not obvious (Lin et al., 2016).

Oxidative stress and DNA damage are important toxic mechanisms of both AFB1 and MC-LR by which carcinogenesis can occur. It has been confirmed that AFB1 can be biotransformed by cytochrome P450 to the reactive 8,9-epoxide (AFBO) which binds to DNA and proteins thus inducing DNA damage (Madrigal-Santillan et al., 2010). Additionally, AFB1 can produce 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of DNA damage induced by oxidative stress, in rats, ducks and woodchucks (Bedard and Massey, 2006). 8-OHdG also produces predominantly G to T transversion mutations in liver which contribute to the process of AFB1 carcinogenesis (Bedard and Massey, 2006). Studies also have shown that AFB1 may induce oxidative damage through the generation of reactive oxygen species (ROS) (Shen et al., 1994), and lead to chromosomal damage through the stimulation of the release of free radicals (Amstad et al., 1984). Similarly, MC-LR was found to induce oxidative DNA damage in hepatoma cells (HepG2) (Chen et al., 2005), and DNA damage induced by MC-LR was either via direct mutagenesis or indirectly through ROS generation, including DNA strand breakage, DNA-protein cross-links, and oxidative DNA base modifications or G-to-T transversions (Zegura et al., 2008). Therefore, oxidative stress and DNA damage are the common hepatic carcinogenesis pathways of AFB1 and MC-LR, however, the genotoxic effects of AFB1 and MC-LR in combination *in vitro* remain largely unknown.

DNA base excision repair (BER) is thought to be the simplest, the most thoroughly defined, and the most important pathway throughout the whole repair process during DNA damage responses caused by oxidative stress (Hazra et al., 2007). Apurinic/apyrimidinic endonuclease 1 (APE1), 8-oxoguanine glycosylase-1 (OGG1) and X-ray repair cross complementing group 1 (XRCC1) are three key DNA repair-related genes involved in BER in mammalian cells (Wang et al., 2016; Hung et al., 2005). APE1, the major AP-endonuclease in mammalian cells, is located on chromosome 14q11.2-q12, has a redox function required for activation of several transcription factors, and protects against oxidative stress (Krokan and Bjoras, 2013). APE1 produces normal 3′-hydroxyl nucleotide termini, which is necessary for DNA repair synthesis and ligation at single- or double-strand breaks, through hydrolyzing the 3′-blocking fragment of oxidized DNA (Peng et al., 2014). OGG1 is located on chromosome 3p26.2 and encodes a DNA repair enzyme that is responsible for the excision of 8-oxo-7,8-dihydroguanine (8-oxoG) and other DNA bases formed by oxidative damage (Paz-Elizur et al., 2008). XRCC1 is located on chromosome 19q13.2 and serves as a scaffold protein for repairing DNA base lesions induced by ROS (Xu et al., 2015). Gene polymorphic variants of XRCC1 have already been reported to interact with AFB1 in the process of HCC in a case-control study (Yao et al., 2014). Here we investigated the effects of AFB1 and MC-LR on liver cells *in vitro,* focusing on the role of BER in the process using assays to measure DNA damage under various combination treatments.

According to the existing research, we hypothesized that combinative exposure to AFB1 and MC-LR leads to enhanced genotoxicity in liver cells *in vitro* through the effects of oxidative stress and dysregulation of DNA base excision repair genes.

2. Materials and methods

2.1. Human hepatic cell lines

The human hepatic cell line (HL7702) was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), and 100 U/ml penicillin and streptomycin (Beyotime, China) in cell culture flasks at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Acute cytotoxicity assay

Cell survival was determined by using the CCK-8 kit as described by the manufacturer (Dojindo, China). Results were drawn with Graphpad Prism 5 software.