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Full Length Research Paper

Cytotoxicity Assessment of Aflatoxin B1 (AFB1) after High Voltage Atmospheric Cold Plasma (HVACP) Generated Reactive Gas Species (RGS) Treatment Using *Artemia salina* and HepG2 Mammalian Cell MTT Bioassays

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Abstract

Mycotoxins are toxic, heat-resistant metabolites of mold and fungi prevalent in food and animal feeds. Recent studies have shown that air passed through high voltage atmospheric cold plasma (HVACP) becomes temporarily ionized, forming reactive gas species (RGS) capable of destroying mycotoxins before reverting to normal air. This study evaluated aflatoxin B1 (AFB1) cytotoxicity with and without RGS treatment using two commonly applied toxicity evaluation model systems: *Artemia salina* [Brine Shrimp Test (BST)] and HepG23-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (HepG2-MTT) *in vitro* bioassays. The BST dose response assay tested 0.25-1.13 µg AFB1/mL. Results obtained showed 11-73% mortality after 72h and 50% mortality (LD_{50}) at 0.6 µg AFB1/mL. Untreated AFB1 (1.7 µg/mL) caused 92% mortality of *Artemia* while RGS-treated AFB1 caused significantly less, 12% (p<.0001). The HepG2-MTT dose response assay tested 0.2-2.8 AFB1 µg/mL, causing 10-32% cell death and 13-57% for 24h and 48h respectively. Untreated AFB1 (0.8 µg/mL) caused 22% and 29% cell death after 24h and 48h while RGS-treated AFB1 caused significantly less, 3% and 2% (p<.05). Overall, this study confirms HVACP generated RGS significantly reduces AFB1 and the AFB1 byproducts formed are less toxic to cells and living organisms.

Keywords: Aflatoxin. High voltage atmospheric cold plasma (HVACP). Reactive gas species (RGS) treatment. Cytotoxicity assessment. HepG2 cell MTT bioassay. *Artemia salina* brine shrimp bioassay.

INTRODUCTION

Aflatoxins are mycotoxins commonly found as contaminants on food and animal feed (Wacoo et al. 2014; Sun et al. 2011; Pankaj et al. 2018). They are predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Ahmed-Adam et al. 2017; Benkerroum 2020). They form a major class of toxic, carcinogenic, and mutagenic compounds for both humans and animals (Ahmed-Adam et al. 2017; Kumar et al. 2017). The compounds cause harmful and acute toxic effects primarily because of damage to the liver (Ahmed-Adam et al. 2017; Benkerroum 2020; Williams et al. 2004). The liver metabolizes the compounds into either reactive epoxide or hydroxylated intermediary products, both of which damage liver cells and potentially lead to tumor formation or acute aflatoxin poisoning known as aflatoxicosis (Sun et al. 2011; Kumar et al. 2017; Williams

et al. 2004). Sub-acute aflatoxin doses covertly suppress immunity making consumers more susceptible to general disease, as well as cause reproductive disorders and growth retardation (Benkerroum 2020, Kensler et al. 2011).

Since aflatoxins are common contaminants in food and feed globally, many international and national government agencies, as well as food processing companies, producers, and traders have set aflatoxin residue standards in food and animal feed products (Williams et al. 2004). For example, the United States' Food and Drug Administration (FDA) has restricted the amount of aflatoxins permitted in human food (<20 ng/g) and animal feed (20-300 ng/g) depending on animal species and age. Seeds, water, fertilizer, energy, money, and livelihoods are commonly wasted because of postharvest losses of cereal grains and other commodities contaminated with these compounds.

For compliance with regulatory guidelines, a variety of physical, chemical, and biological strategies are in demand for partial or complete elimination of mycotoxins from food and feed (Pankaj et al. 2018; Karlovsky et al. 2016). Several methods like alkalization, ammoniation, organic acids, and heat or gamma radiation, have been explored as post-harvest intervention strategies to minimize aflatoxins in numerous agricultural products (Pankaj et al. 2018; Williams et al. 2004; Karlovsky et al. 2016). However, these technologies may compromise food and feed quality and leave undesirable residues.

NanoGuard Technologies (Saint Louis, MO) has developed an alternative non-thermal treatment process that reduces mycotoxins on contaminated food and feed. The technology uses a patented high voltage atmospheric cold plasma (HVACP) system to ionize a working gas (air and/or modified atmospheres) and form reactive gas species (RGS), capable of reducing mycotoxins as well as microorganisms including bacteria, viruses, molds, and fungi. The working gas and electricity are the feedstocks for the treatment. RGS, the non-equilibrium species created after ionization of the gas during its passing through the HVACP generator, contains reactive nitrogen species (RNS) and reactive oxygen species (ROS). Although ozone consistently forms in the process when oxygen is present in the working gas (for example from O_2, H_2O, or CO_2) the properties and reactions of the RGS are not explained by the presence of ozone alone (Hochwalt and Keener, 2021).

Plasma systems tailored for ozone generation have been used to purify wastewater since 1907 (Hussain et al. 2022). Since then, plasma has been successfully applied for a myriad of purposes, including lighting, air purification, medical procedures, and surface modifications (Kim et al. 2023). Additionally, a rising number of contemporary studies have demonstrated that plasma can reduce a wide range of contaminants on a wide range of materials, including mycotoxins on food and feed (Zhang et al. 2023). This particular area of research has garnered much interest in the past decade especially because of concerns regarding rapid population growth, global food security, and increased knowledge of mycotoxin-driven injury to humans and animals.

Because plasma treatments may differ substantially per type (in-field, in-package, or remote plasma treatments), system, and commodity, each specific application must be substantially evaluated to ensure efficacy and commercial feasibility. Aflatoxin B1 (AFB1) is the most potent and pervasive aflatoxin, so it has historically been the most studied (Kensler et al. 2011). Recent studies of in-field and in-package plasma treatments of chemically isolated AFB1 elucidated similar, generally oxidized degraded byproducts as determined by nuclear magnetic resonance spectroscopy and/or mass spectrometry (Shi et al. 2017; Hojnik et al. 2021). The AFB1 byproducts generated by these direct plasma treatment systems were determined to be significantly less toxic than AFB1 to HepG2 cells (Nishimwe et al. 2021; Hojnik et al. 2021). The current study investigated if RGS treatment of AFB1 also reduces its cytotoxicity, alongside concurrently observed RGS treatment reductions of AFB1 genotoxicity (Ndengele et al. 2023).

Thus, the *Artemia salina* bioassay [or Brine Shrimp Test (BST)] and HepG2-MTT cell cytotoxicity assay were used to evaluate the toxicological effects of AFB1 with and without RGS treatment. NanoGuard's prototype plasma generator was scaled 20x from lab- to pilot-scale. The lab-scale device was used to treat AFB1 in the *Artemia* study and the pilot-scale device for the HepG2-MTT study.

Artemia salina have been widely used for *in vitro* toxicology bioassays for over 50 years to assess the toxicity of many chemicals and environmental pollutants (Ntungwe et al. 2020). *Artemia* provide a quick and convenient *in vitro* biological assay system to evaluate the bioactivity and toxicity of various chemical molecules (Banti and Hagdjkakou 2021; Ntungwe et al. 2020). Because of its reliability and low cost, the *Artemia* bioassay is a scientifically accepted toxicity monitoring system for pharmaceutical products and a wide range of chemical compounds (Neu et al. 2014; Libralato et al. 2016). It has already been widely used for the toxicological evaluation of AFB1 and other mycotoxins, demonstrating its suitability for evaluating the biosafety of RGS-treated and -untreated AFB1 (Wang et al. 2016; Iram et al. 2016). A dose-response bioassay was run to confirm the trend of increased mortality with increased AFB1 concentration, as well as to ensure a lack of toxicity from the DMSO present in the media used for toxin extraction and solubilization. Comparative toxicity assessment of AFB1 vs. RGS-treated AFB1 (degradants) was also performed.

Similarly, HepG2 human liver cells were used to investigate the cytotoxicity of AFB1 and its RGS-reacted

degradants using the tetrazolium salt, (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) standard assay (Sylvester 2011; Al-Qubaisi et al. 2011; Senthilraja and Kathiresan 2015). The MTT assay is a colorimetric assay used for determining cell viability based on their metabolic activities. Within living cells, the nicotinamide adenine dinucleotide phosphate (NADPH) dependent intracellular oxidoreductase enzymes in mitochondrial cellular compartments determine metabolic activities and reduce soluble yellow MTT dye into insoluble purple formazan crystals. This activity reflects the number of viable and metabolizing cells present in a system. In short, the *in vitro* assay quantifies the reduction of MTT dye into purple formazan crystals caused by metabolically active and viable cells. When solubilized with organic solvents, the crystals produce a colored solution. The color intensities of these solutions are correlated to cytotoxicity (as measured by cell death) towards mammalian cells due to toxic materials or agents through comparison to healthy cells from the same cell line incubated without the potentially toxic substance (Sylvester 2011; Senthilraja and Kathiresan 2015). Usually, small amounts of living metabolizing cells reduce small amounts of MTT dye causing less color intensity, and large amounts of live cells tend to show high rates of MTT reduction marked by a high intensity of solubilized formazan crystals, hence more color. Measuring the absorbance of these solutions using a spectrophotometer at wavelengths between 500-600 nm provides a reliable method of determining the number of viable cells in a living system (Senthilraja and Kathiresan 2015; Fotakis and Timbrell 2006).

MATERIALS AND METHODS

Materials

Premium grade *Artemia salina* eggs were purchased from “Brine Shrimp Direct” (Ogden, Utah) to produce the *Artemia* larvae for the study. The following brine shrimp test reagents and supplies were purchased from various general stores: baking soda, Top Fin® 5-gallon air pump, Tetra® EasyStrips™ (Blacksburg, VA), a desk lamp, 56-watt bulbs, Instant Ocean® Sea Salt (Blacksburg, VA), and a 2-liter container. Cell culture flasks, 48-well plates, 96-well plates, and 100 mm petri-dishes were purchased from Midwest Scientific (Fenton, MO). The HepG2 cell line was purchased from Sigma-Millipore (St. Louis, MO). The Eagle’s Minimum Essential Medium (EMEM: 4.5 g/L glucose and L-Glutamine) for cell growth was purchased from American Type Culture Collection (ATCC, Manassas, VA). Other medium reagents, Streptomycin, Penicillin, Fetal Bovine Serum (FBS), Trypsin-EDTA (0.25% trypsin/0.53 mM EDTA in HBSS (without calcium and magnesium) were also purchased from ATCC. The Phosphate-buffered saline (PBS) was purchased from Corning Cellgro (Glendale, AZ). MTT dye was from Thomas Scientific (Swedesboro, NJ). The microplate reader was from Molecular Devices (San Jose, CA). The 90 mm diameter Whatman™ Grade 5 (2.5 µM) filter papers were purchased from Amazon.com (Seattle, WA) and purified crystalline aflatoxin B1 was purchased from Cayman Chemical Company (Ann Arbor, MI). Methanol and Dimethyl Sulfoxide (DMSO) were purchased from VWR (Radnor, PA).

Methods

Culturing Artemia salina Eggs

One liter of artificial sea water (brine) was prepared by dissolving 35 g of Instant Ocean® Sea Salt plus 150 mg baking soda into one liter of dH2O in a continuously aerated 2L plastic bottle screwed into the tank’s base. The air pump was connected to the base of the bottle with ¼’’ vinyl tubing. The pH was checked with a Tetra® EasyStrip™ and maintained between 8.2-8.4 using additional baking soda if required. One tsp of premium grade *Artemia salina* eggs (cysts) were then added to the prepared brine, and a 56-watt light bulb lamp was positioned 4 inches from the bottle for continuous heating and illumination. Below is the culturing apparatus used for this study (Fig. 1).

After 24h incubation at room temperature (RT), 90% of the cysts hatched into their larvae stage. This larvae suspension (25 mL) was pipetted out for the *Artemia* bioassays.

Preparation of AFB1-Containing Filter Papers, RGS Treatment and Extract Preparation

To treat AFB1 with RGS at concentrations relevant for these toxicity assays, crystalline AFB1 dissolved in methanol was adsorbed onto filter papers and allowed to dry.

For the *Artemia* assay, two filter papers were contaminated with 90 µg of AFB1 per filter paper. One AFB1 contaminated filter paper was treated with RGS for 80 min, and the second filter paper stayed untreated to serve as untreated (positive) control. Both the RGS-treated and positive control filter papers were separately extracted using 7 mL of 12% DMSO in *Artemia* media. The extraction process for each paper consisted of 90 seconds of agitation (shaking and vortexing), 45 minutes of RT incubation, and 90 more seconds of agitation. Each extract was then centrifuged at 15,000 x g to settle all paper particles, and the clean supernatants were then transferred to new tubes and AFB1 concentrations determined by HPLC using a modified AOAC method (AOAC #994.08) (Trilogy Analytical Lab, Washington, MO).

For the MTT assay, six papers were contaminated with 241 µg of AFB1 per filter paper. Half were treated with RGS for 90 min while the rest stayed untreated to serve as positive control. Fig.2a below shows the plasma

Fig.1. *Artemia salina* Culturing Apparatus.

a **b** **c**

Fig.2 a, b, and c) Cold plasma chamber where ionization of air occurs and RGS produced (close-up view while operating); **b)** AFB1-contaminated filter paper discs clamped in place in the contacting chamber; **c)** Sealed contacting chamber during RGS-treatment.

chamber where RGS was produced from air. Approximately 20 meters from the plasma chamber, filter paper discs were clamped for treatment in the sealed contactor. Fig.2b and 2c show the contactor where discs were treated with RGS.

After treatment, the six papers were separately extracted using 2x4 mL washes of 6% DMSO in dH2O. The extraction process for each paper consisted of 90 seconds of agitation (shaking and vortexing), 5 minutes of RT incubation, and 90 more seconds of agitation. The first 4 mL wash was removed before the second 4 mL wash was conducted identically, except with a 30 min incubation period. Each extract was then centrifuged at 15,000 x g to settle all paper particles, and the clean supernatants were then transferred to new tubes and the AFB1 concentration was determined using HPLC as described above.

AFB1 Dose Response Artemia Bioassay and LD_{50} Determination

A 48-well bioassay plate was used for the dose response evaluation. The untreated, positive control filter paper extract obtained as described above was used as the AFB1 stock solution. Six AFB1 concentrations ranging from 0.25 µg/mL to 1.13 µg/mL were used in the study to develop the dose response curve. Two sets of negative controls (wells with no AFB1) were also analyzed with this study. The “Negative Control” *Artemia* wells contained medium with no DMSO while the “Vehicle

Control" wells contained medium with DMSO. The purpose of the DMSO was to evenly distribute the AFB1 in the liquid medium.

The volume of AFB1 or control solution in each well was 150 µL. *Artemia* larvae suspension (150 µL) containing roughly 15-35 larvae was then added to each well resulting in a total volume of 300 µL in each well. The final DMSO concentrations in the Vehicle Control and AFB1 wells were maintained at 4%. The final AFB1 concentrations in the test wells were 0.25 µg/mL, 0.43 µg/mL, 0.60 µg/mL, 0.78 µg/mL, 0.95 µg/mL, and 1.13 µg/mL. A minimum of 6 wells per control or AFB1 test solution was used.

The person dispensing the solutions into the wells created a different well legend schematic for each assay, and this legend was hidden from the individual counting the dead larvae to reduce risk of bias. The well plate was incubated at RT (25^{o}C) for 72h. After the incubation period, the dead *Artemia* larvae in each well were counted using a microscope. Next, the living larvae in each well were immobilized by adding methanol prior to counting total larvae per well. The total larvae count for each well was then used to calculate percentage mortality and the resulting mortality data was used to develop the dose response curve and LD_{50} of AFB1 for *Artemia* larvae.

Artemia Bioassay of RGS-Treated AFB1 vs. Untreated AFB1 (Comparative Assessment)

The RGS-treated AFB1 filter paper extract and its corresponding positive control extract were used to evaluate the toxicity of AFB1 with and without RGS treatment. Both extracts were identically diluted with media to the final DMSO concentration of 8%. The RGS-treated extract, the positive control extract, and a toxin-negative DMSO+media vehicle control solution were then transferred to a 48-well plate. After addition of 150 µL of *Artemia* larvae, the final DMSO concentration was 4%. After all dilutions, the final concentration of AFB1 in RGS-treated extract wells (test) was 0.2 µg AFB1/mL + 1.5 µg AFB1 degradants/mL. The final concentration of AFB1 in the RGS-untreated extract wells (positive control) was 1.7 µg AFB1/mL. A minimum of 8 wells per control or test solution were used. Like the dose response assay, the person dispensing the solutions into the wells created a different well legend schematic for each assay, and this legend was hidden from the individual counting the dead larvae to reduce risk of bias. The experiment was repeated two additional times.

MTT Cytotoxicity Assay HepG2 Cell Culture

The HepG2 cells were grown exponentially in either plastic tissue culture plates or flasks. To start the cell culture, cells frozen in DMSO were thawed out and transferred into a 15 mL tube containing 10 mL of EMEM medium without fetal bovine serum (FBS). The cells were centrifuged at 500 x g for 5 min and the supernatant was removed and discarded. The cells were then resuspended with 15 mL of complete medium containing 10% FBS and transferred into a 75 mL tissue culture flask. Complete culture medium also contained antibiotics (100 I.U./mL penicillin and 100 µg/mL streptomycin) to avoid potential microbial contamination of the culture. The flasks with cells were incubated at 37°C in a humidified 5% CO_2 incubation chamber. Cells were allowed to attach to the flask floor and grow to 70% confluence before use in experiments. For the MTT experiments, cells were grown in complete medium in 96-well plates. A minimum of 8 wells per condition was used for each blank, control, or test extract.

MTT Assay Extract Preparation

The MTT cytotoxicity assays were performed using AFB1 extracts obtained from both RGS-treated and untreated filter papers. Briefly, six filter paper discs were spiked by spotting each with 1.2 mL of AFB1 in 100% methanol solution (201 µg/mL) and allowed to dry. The AFB1-spiked filter paper discs were then randomly sorted into two groups of three each. One group served as the positive control (not treated with RGS) and the other was the test (treated with RGS for 90 min). After treatment, each filter paper disc was cut into small pieces into respective 15 mL vials and extracted twice (2x) with 6% DMSO-dH2O (4 mL for each extraction). The two 4 mL extracts of each filter paper were pooled together in a clean tube and supernatants recovered after centrifugation at 15,000 x g for 5 minutes. The resulting supernatants were then used for HepG2-MTT assays. For MTT experiments, cells were grown in complete medium in 96-well plates. Two types of MTT bioassays were performed: AFB1 dose response assay and comparative assessment of RGS-treated and untreated AFB1.

AFB1 Dose Response HepG2-MTT Assay

The dose-response AFB1 stock solution was prepared by dissolving crystalline AFB1 into 6% DMSO-dH2O to a final concentration of 55 µg AFB1/mL. Appropriate amounts of this stock solution were diluted with serum-free cell medium and used in the MTT assay. The final AFB1 concentrations in test wells were 0.2, 0.3, 0.8, 1.7, and 2.8 µg/mL with a final DMSO concentration of 0.3%. The total volume of assay solution in each well was 200 µL. A minimum of 8 wells per control or AFB1 test solution was used in each 96-well plate.

The AFB1 dose-response MTT assay was conducted to evaluate cytotoxicity effects of AFB1 at various concentrations (dosage) on HepG2 cells. Briefly, the HepG2 cells were plated in 96-well plates at a seeding density of $5x10^4$ cells per well in 200 µL of culture medium and grown overnight before AFB1 exposure.

Before starting the experiment, the medium in the wells was replaced with AFB1 solutions (200 µL) of appropriate concentrations in 0.3% DMSO serum-free medium (8 wells per concentration per plate). There were also three sets of control wells without AFB1. Blank control wells (8 per plate) contained medium with no cells and were used to establish the OD baseline. Negative control wells contained cells in 0% DMSO medium (16 per plate). Lastly, the vehicle control wells contained cells in 0.3% DMSO medium and were used as the normalizing control (cells at 100% viability, 16 per plate).

An OD comparison of vehicle to negative control wells was used to confirm that the 0.3% DMSO present in the assay solution did not impact cell viability. The plates were then incubated for 24h or 48h in a 5% CO_2 incubator before addition of MTT. After the respective incubation period, the medium was removed from the adherent cells and 50 µL of fresh media without FBS was added to each well followed by 50 µL of MTT solution [5 mg/mL in phosphate buffered saline (PBS-1X)]. The plate was further incubated in a humidified chamber for 3h. After incubation, 150 µL of MTT solvent (acidified isopropanol) was added to each well. The plate was then wrapped in foil and shaken at room temperature (RT) for 15 min on a flat rotary shaker. The resulting formazan crystals were further solubilized by pipetting the well solutions up and down several times until color became uniform. The optical density (OD) of the formazan solution was measured at 590 nm within 1h of solubilization. The color intensity of the dissolved crystals was used to determine cell viability, integrity, and AFB1 cytotoxicity to HepG2 cells (Vega-Avila and Pugsley 2011). Three experiments were conducted, each in duplicate (i.e., two 96-well plates per experiment).

HepG2-MTT Comparative Assay of Control and Test Extracts

The HepG2 cells used for this comparative study were also grown in 96-well plates as described above for the dose response assay. The three control (AFB1 recovered from filter discs not treated with RGS) and three test (AFB1 + degradants recovered from RGS-treated filter discs) extracts were used for study. To prepare the six extracts for plating with cells, each 6% DMSO-dH2O filter paper extract was diluted 20x with serum free cell medium to a final DMSO concentration of 0.3% in wells. Each extract was plated with HepG2 cells into an 8-well column within each 96-well plate. The plates were then incubated for 24h and 48h as described in the AFB1 dose response study. Similar to the dose response assay, this assay also included a set of blank control wells (media with no cells), negative control wells (cells in 0% DMSO media) and vehicle control wells (cells in 0.3% DMSO-dH2O media). The vehicle control wells were again used as the normalizing control (cells at 100% viability) for determining the cytotoxicity of the studied extracts. A minimum of 8 wells per filter paper extract or control solution was used in each 96-well plate. The comparative assessment assay was performed in duplicate (two experiments, one 96-well plate per incubation period per experiment).

Statistical Analysis

Statistical analysis was performed after collaboration with a professional bioassay statistician (Statistical Consultants Plus, LLC, St. Louis, MO). Arcsine correlation was used to transform the raw data expressed in percentage mortality (*Artemia*) or average percentage cytotoxicity (HepG2 MTT) into regression proportions to reduce noise. T-test analyses were then performed. For the comparative *Artemia* assays, $\alpha = 0.0001$ was the significance cut-off point (two-tailed, equal variance) while $\alpha = 0.05$ was used for the HepG2-MTT assays (one-tailed, equal variance). Error bars and ± signs in MTT figures and tables represent one standard error from the mean (SEM) in each direction. Error bars and ± signs in *Artemia* figures and tables represent one standard deviation in each direction.

RESULTS

HPLC AFB1 Analysis Results for Filter Paper Extracts

The results from HPLC analysis for AFB1 of the filter paper extracts were corrected for dilutions and their undiluted concentrations are shown below in Table 1. Two total papers were analyzed for the *Artemia* study (one per group) and six (three per group) for the HepG2 MTT study. AFB1% reduction due to RGS treatment is displayed as well as % recovery. The equations used for calculating AFB1 reduction from RGS treatment and recovery from filter paper extraction are as follows:

$$\text{Reduction \%} = \left(1 - \frac{\text{Treated Paper Extract AFB1 Concentration}}{\text{Untreated Paper Extract AFB1 Concentration}}\right) \times 100\%$$

$$\text{Recovery \%} = \frac{(\mu\text{g/mL AFB1 recovered in extract}) \times (\text{mL of extract})}{\text{Starting AFB1 } (\mu\text{g})} \times 100\%$$

Dose Response Effects of AFB1 on Artemia salina: LD_{50} Determination

To determine the toxicity in *Artemia salina* larvae from various concentrations of AFB1, a dose response assay was conducted with larvae mortality calculated after 72h of exposure to toxin. The following data in Table 2 and Fig. 3 indicated that *Artemia salina* larvae mortality increased with increasing AFB1 concentration.

Table 1. HPLC AFB1 Analysis Results for Filter Paper Extracts.

Bioassay	Filter Paper Extract Description	AFB1 Conc.[a] (µg/mL)	AFB1 Recovery (%)	AFB1 Reduction (%)
Artemia	Untreated AFB1	10.2	79.3%	na[b]
	RGS-Treated AFB1	1.2	9.3%	87.8%
HepG2 MTT[c]	Untreated AFB1	16.5 ± 1.0	54.8 ± 3.3%	na[b]
	RGS-Treated AFB1	1.9 ± 0.3	6.3 ± 0.9%	88.6 ± 1.7%

[a]AFB1 concentrations determined by HPLC using a modified AOAC method (AOAC #994.08) at accredited mycotoxin testing lab (Trilogy Analytical Lab, Washington, MO).
[b]not applicable
[c]average ± SEM

Table 2. AFB1 Dose Response *Artemia* Bioassay Results.

Sample Description	Percentage Mortality after 72h ± SD
0% DMSO Negative Control	2.4 ± 3.7%
4% DMSO Vehicle Control	4.0 ± 4.8%
0.25 µg/mL AFB1	10.9 ± 8.5%
0.43 µg/mL AFB1	26.0 ± 16.8%
0.60 µg/mL AFB1	54.0 ± 15.8%
0.78 µg/mL AFB1	64.1 ± 8.8%
0.95 µg/mL AFB1	72.7 ± 10.2%
1.13 µg/mL AFB1	73.0 ± 16.8%

Fig.3 is a visual representation of the dose response curve of AFB1 to *Artemia* (fit with a Four Parameter Logistic curve). To fill out the high-concentration end of the curve, the data set in Table 2 was expanded to include the RGS-untreated positive control results from the Comparative Assessment described in the following section (Table 3). The R^2 value of the curve was 0.986. As seen in both Table 2 and Fig.3, the mortality of *Artemia salina* larvae increased with AFB1 concentrations ranging from 0 to 1.13 µg AFB1/mL, and the LD_{50} concentration

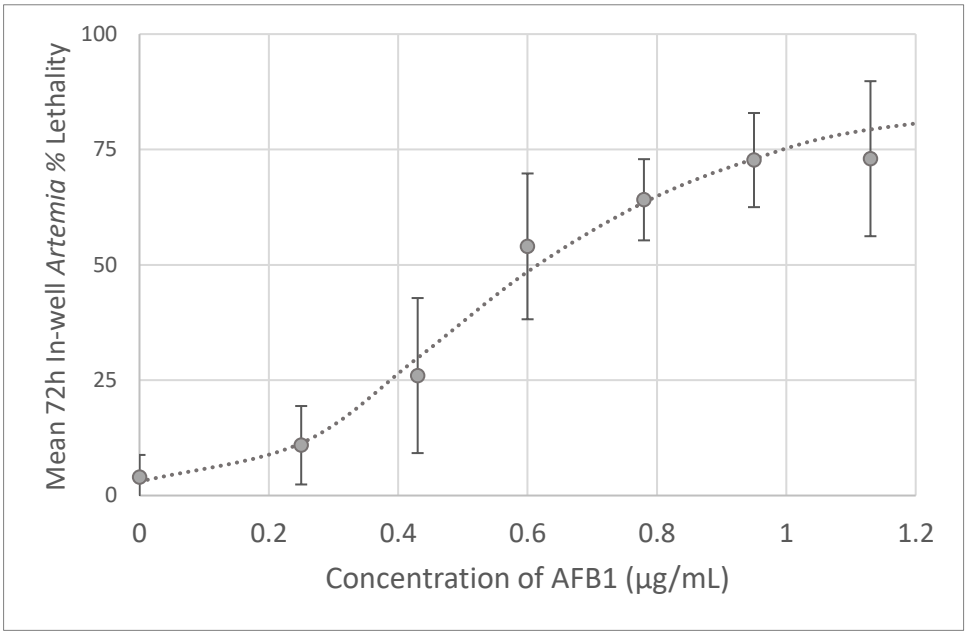

Fig.3 Mortality of *Artemia salina* vs. AFB1 Concentration.

Table 3. Comparative *Artemia* bioassay of Untreated vs. RGS-Treated AFB1.

Sample Description	In-well Concentration (µg/mL)	*Artemia* Percentage Mortality ± SD		
		Expt 1	Expt 2	Expt 3
4% DMSO Vehicle Control	0	2.9 ± 3.7%	14.1 ± 8.4%	5.8 ± 5.9%
Untreated AFB1 (Positive Control)	1.7 AFB1	92.0 ± 5.9%	95.4 ± 4.0%	87.9 ± 11.4%
RGS-Treated AFB1 (Test)	0.2 AFB1 +1.5 Degradants	14.3 ± 8.8%	14.1 ± 8.4%	4.8 ± 3.8%

of AFB1 to *Artemia salina* was determined to be 0.6 µg/mL. The 4% DMSO vehicle control wells did not indicate a significant difference in *Artemia* mortality when compared to the negative control wells (p>.5).

***Artemia* Bioassay of Untreated vs. RGS-Treated AFB1 (Comparative Assessment)**

To determine whether RGS treatment reduces the toxicity of AFB1, an *Artemia* comparative bioassay was conducted using the extract from one RGS-treated filter paper and its corresponding untreated positive control. *Artemia* mortality was determined to compare the toxicity of equal volumes of both extracts (RGS-treated and untreated) under identical dilutions and 72h incubation. The final AFB1 concentration in positive control wells was 1.7 µg AFB1/mL, while the RGS-treated extract wells contained 0.2 µg/mL AFB1 + 1.5 µg/mL AFB1/RGS byproducts. The arcsine-normalized *Artemia* mortality percentages observed after incubation of the two test and control extracts were compared for each of three experiment.

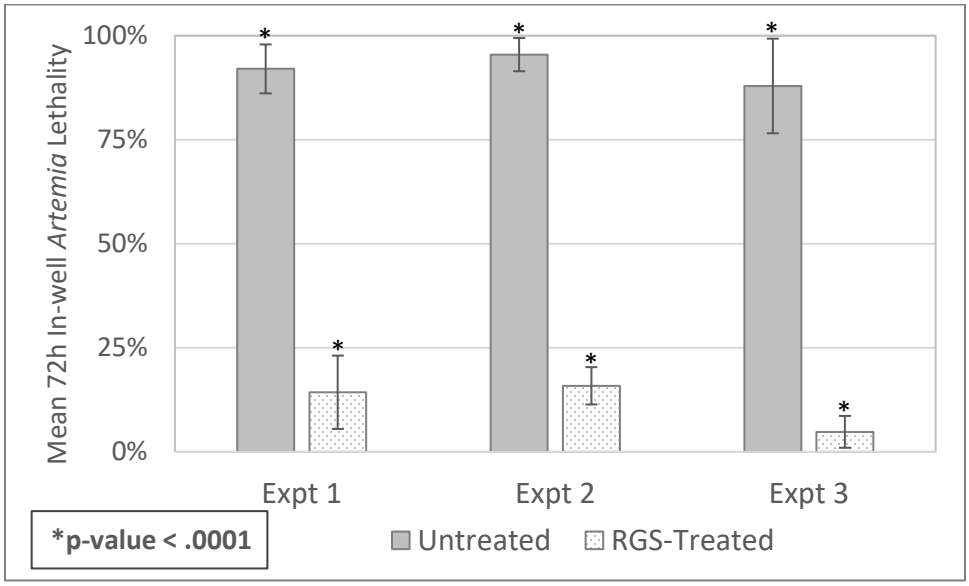

Fig.4 Comparative *Artemia* bioassay of Untreated vs. RGS-Treated AFB1.

Table 4. HepG2 Cytotoxicity Percentage at Varying AFB1 Concentrations (24h and 48h Incubation)[a]

Column Description (In-well Conc.)	24h Avg ± SEM	48h Avg ± SEM
Negative Control[b]	2 ± 2	3 ± 1
Vehicle Control[c]	2 ± 2	0 ± 0
0.2 µg/mL AFB1	10 ± 4	13 ± 1
0.3 µg/mL AFB1	10 ± 4	19 ± 3
0.8 µg/mL AFB1	15 ± 3	35 ± 4
1.7 µg/mL AFB1	23 ± 3	48 ± 5
2.8 µg/mL AFB1	32 ± 4	57 ± 4

[a]data from three experiments, two 96-well plates per incubation period per experiment.
[b]cells plus media, no AFB1 and no DMSO.
[c]cells plus 0.3% DMSO media, no AFB1.

Results from the comparative study are shown below in Table 3 and Fig.4.

Artemia mortality from untreated AFB1 (positive control) was significantly higher than RGS-treated AFB1 (test). Across three experiments, AFB1 caused 87.9-95.4% mortality while the RGS-treated AFB1 caused 4.8-14.3%. The bioassay results demonstrated a significant ($p<0.0001$) reduction of cytotoxicity and death from HVACP generated RGS treatment.

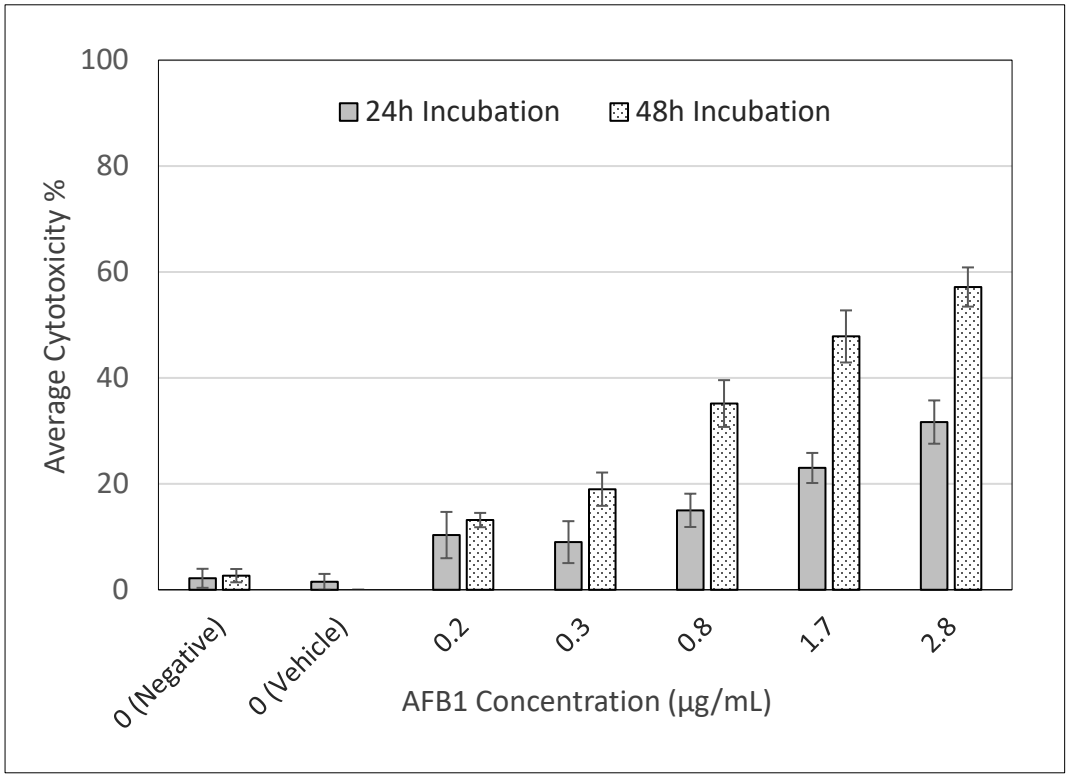

Fig.**5** HepG2 Cytotoxicity Percentage at Varying AFB1 Concentrations.

Dose-dependent AFB1 Cytotoxicity Effects on HepG2 Cells

The cytotoxic effects of AFB1 and RGS-treated AFB1 on the mammalian HepG2 cell line were also investigated using the MTT assay. This *in vitro* assay is a widely accepted indirect measurement of cell viability for many types of mammalian cells before and after exposure to potentially hazardous substances (Sylvester 2011; Senthilraja and Kathiresan 2015). The average OD of each control and test extract was normalized to the vehicle control to determine cell viability. The HepG2 cytotoxicity percentage was then calculated by subtracting the cell viability from 1 and multiplying that difference by 100% as shown below.

$$\text{Cytotoxicity} = \left(1 - \frac{\text{Average OD(Sample Wells)}}{\text{Average OD(Vehicle Control Wells)}}\right) \times 100\%$$

The data obtained showed a dose dependent AFB1 cytotoxic effect on HepG2 cells (See Table 4 and Fig.5). In other words, HepG2 cell viability decreased as AFB1 concentration increased, resulting in increased cytotoxicity percentage. The mean percentage of cytotoxicity and the SEM were calculated from the data.

Comparison between the 0% DMSO Negative Control wells and the 0.3% DMSO Vehicle Control wells confirm that the 0.3% DMSO used in the assay did not interfere with cell viability during both 24h and 48h incubations.

MTT Assay of AFB1 Before and After RGS Treatment Using HepG2 Cells

The cytotoxicity studies of the AFB1 filter paper extracts on HepG2 cells using the MTT assay revealed that RGS-treated AFB1 is less toxic than untreated AFB1 for both 24h and 48h incubations. The data from the 24h and 48h incubations are presented in Table 5 as well as in Fig.6a and 6b. No toxic effects were observed for the 0.3% DMSO vehicle control wells relative to the negative control wells.

The data showed that RGS-treatment significantly ($p<.05$) reduced the cytotoxicity of AFB1 towards HepG2 cells for both incubation periods. The extract from RGS-treated AFB1 resulted in toxicity levels nearly identical to the negative and vehicle control groups. The results from the study demonstrated that RGS-treated AFB1 is non- or less toxic ($p<.05$) to HepG2 cells compared to untreated AFB1.

Table 5. Untreated vs. RGS-Treated AFB1-contaminated Filter Papers: 24h and 48h MTT Results[a]

Column Description	Experiment 1 Cytotoxicity % (24h)	Experiment 2 Cytotoxicity % (24h)	Experiment 1 Cytotoxicity % (48h)	Experiment 2 Cytotoxicity % (48h)
Negative Control[b]	3	3	0	3
Vehicle Control[c]	0	0	0	0
Untreated A	26	10	18	37
Untreated B	25	23	31	33
Untreated C	18	31	25	31
RGS-Treated A	3	1	0	0
RGS-Treated B	0	4	7	3
RGS-Treated C	0	7	0	0
Untreated Avg[d]	22.7 ± 2.5	21.6 ± 6.0	24.7 ± 4.0	33.6 ± 1.8
RGS-Treated Avg[e]	1.0 ± 1.0	4.2 ± 1.6	2.4 ± 2.4	0.9 ± 0.9

[a]each experiment comprised one 96-well plate per incubation period.
[b]cells plus media, no AFB1 and no DMSO.
[c]cells plus 0.3% DMSO media, no AFB1.
[d]untreated average in-well concentration = 0.8 µg/mL AFB1
[e]treated average in-well concentration = 0.1 µg/mL AFB1 + 0.7 µg/mL degradants

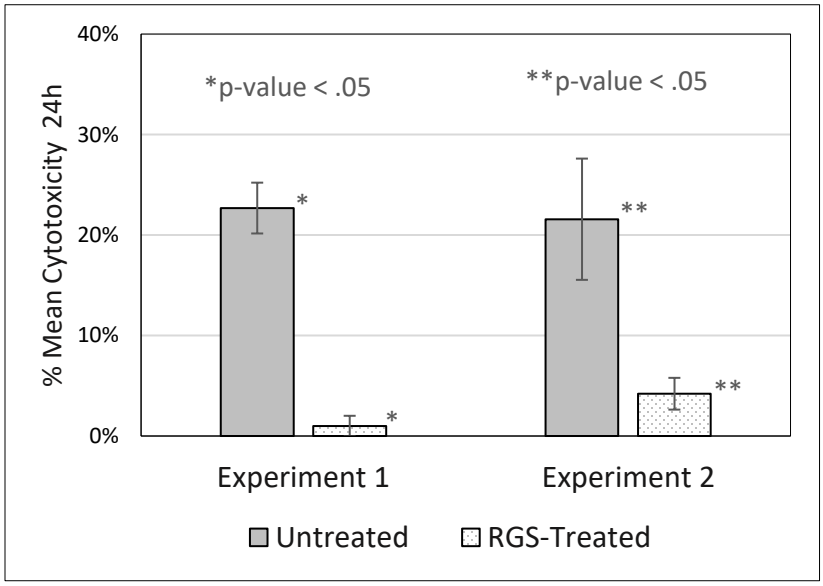

Fig.6a HepG2 Cytotoxicity Percentage of AFB1 and RGS-Treated AFB1 (After 24h Incubation).

DISCUSSION

A proprietary gaseous treatment has been developed with the capability of reducing AFB1 and other mycotoxins on food, feed, and agricultural commodities (Hochwalt and Keener 2021). Though the process reliably reduces AFB1 as quantified by HPLC standard analytical methods, it was unknown if the AFB1 degradants resulting from the treatment cause toxicity to living organisms and cells. The purpose of this study was

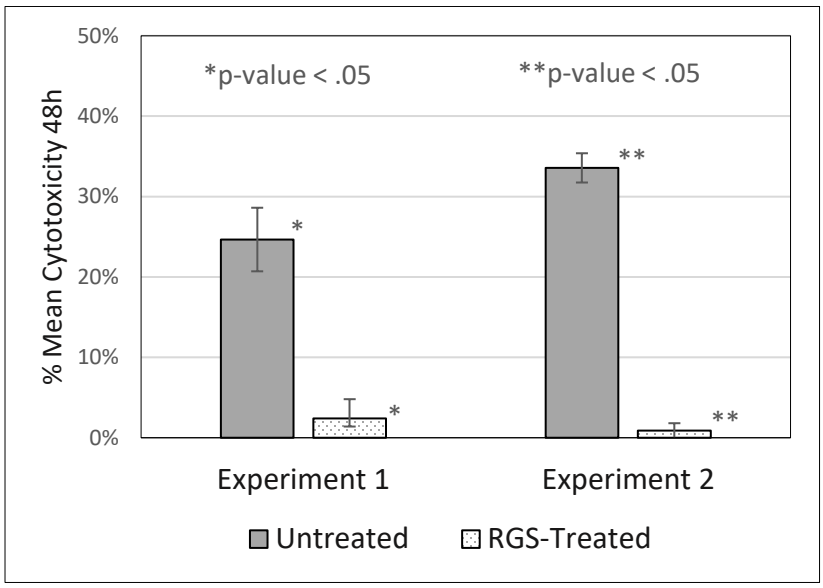

Fig.6b HepG2 Cytotoxicity Percentage of AFB1 and RGS-Treated AFB1 (After 48h Incubation).

to use *Artemia salina* and HepG2-MTT bioassays on HepG2 cells to evaluate the toxicity of AFB1 and the AFB1 degradants produced by the RGS treatment.

The *Artemia* larvae bioassay is a commonly accepted cytotoxicity monitoring system for a wide range of chemicals including pharmaceuticals and environmental pollutants (Banti and Hadjikakou 2021; Ntungwe et al. 2020; Hamidi et al. 2014). Similarly, the *in vitro* MTT assay is also widely used to gauge the toxicity of potentially hazardous chemicals through the indirect measurement of cell viability following incubation with many types of mammalian cells (Sylvester 2011; Senthilraja and Kathiresan 2015).

Two types of bioassays were conducted for both *Artemia salina* and HepG2 cells. The first, a dose response bioassay, was performed to determine the dose dependency of AFB1 towards each model system. For *Artemia*, mortality increased with increasing concentration of AFB1 and the LD_{50} was determined to be 0.6 µg AFB1/mL. The AFB1 concentrations in the dose response bioassay ranged from 0.25-1.13 µg AFB1/mL and the larvae mortality ranged from 10.9 ± 8.5% to 73.0 ± 16.8%. Minimal *Artemia* mortality was observed in the negative and vehicle controls, 2.4 ± 3.7% and 4.0 ± 4.8% (Table 2), respectively.

The HepG2-MTT dose response assay tested AFB1 concentrations ranging from 0.2 to 2.8 µg/mL. The results showed cytotoxicity percentages ranging from 10 ± 4% to 32 ± 4% and 13 ± 1% to 57 ± 4% after 24h and 48h incubation, respectively. Like the *Artemia* study, all negative controls maintained good cell viability (at both incubations) throughout the duration of the study, and toxicity increased as AFB1 concentration increased (Table 4, Fig.5), with in-well concentrations of 0.2 µg AFB1/mL and higher causing less cell viability than the negative or vehicle controls.

The second type of bioassay was a comparative assessment which directly compared mortality caused by untreated AFB1 vs. AFB1 treated with HVACP generated RGS. *Artemia* mortality in the untreated AFB1 wells was much higher than in the RGS-treated AFB1 wells. The mortality rates of both test and positive controls were compared to each other by statistical analysis of the arcsine-normalized mortality percentages. There was significantly lower ($p<0.0001$) *Artemia* mortality caused by the RGS-treated AFB1 extract compared to its corresponding untreated AFB1 extract (Table 3, Fig. 4).

HepG2 MTT assays comparing the cytotoxicity of RGS-treated AFB1 to untreated AFB1 were also performed (Table 5, Fig.6a and 6b). After 24h of incubation, the average cytotoxicity percentages due to the untreated, positive control extract (1.7 µg AFB1/mL) were 22.7 ± 2.5% and 21.6 ± 6.0% for the two experiments (three filter paper extracts per group per experiment) which correlated well with the dose response results. Similarly, in the 48h incubation, the percentage cytotoxicity for the positive control extract was 24.7 ± 4.0% and 33.6 ± 1.8% respectively, also corresponding well with the dose response data. On the other hand, after 24h incubation, the average cytotoxicity percentages for the test extract (RGS-treated AFB1, 1.5 µg AFB1 degradants/mL + 0.2 µg unreacted AFB1/mL) were 1.0 ± 1.0% and 4.2 ± 1.6%. After 48h incubation, the percentage cytotoxicity for the RGS-treated extract was 2.4 ± 2.4% and 0.9 ± 0.9%, respectively. These results indicated that RGS treatment significantly ($p<.05$) reduced

the cytotoxicity of AFB1 to HepG2 cells.
It is well known that AFB1 is very toxic to mammalian cells and causes hepatic damage as well as liver cancer (Ahmed-Adam et al. 2017; Kumar et al. 2017; Kensler et al. 2011). There is also evidence of the mycotoxin's ability to induce cell death (apoptosis) in different cell types (Benkerroum 2020; Vega-Avila and Pugsley 2011). The data from this study agrees with previously published findings, confirming that intact AFB1 is cytotoxic to *Artemia salina* and mammalian cells, specifically HepG2 (Ahmed-Adam et al. 2017; Benkerroum 2020). More notably, both toxicity screening biological systems in this study also demonstrated that HVACP generated RGS degrades AFB1 into significantly less or nontoxic byproducts, agreeing with similar contemporary studies (Hojnik et al. 2021; Ndengele et al. 2023). Based on these results and correlations observed in toxicity screening literature, the degradants produced by the AFB1/RGS reaction are likely less toxic than AFB1 in higher living organisms such as fish, avians, and mammals (Parra et al. 2001).
Additional research may be done to further incentivize commercial adoption of this technology. Specific commodities could be treated and studied to 1) prove HVACP generated RGS treatment reduces mycotoxins without negatively impacting quality, 2) demonstrate it reduces the toxicity of other mycotoxins, and 3) prove it sustainably scales with commercially acceptable costs.

CONCLUSION

Mycotoxins contaminate global food supplies and burden many lives. HVACP generated reactive gas species (RGS), offer a promising non-thermal post-harvest treatment to reduce the prevalence and toxicity of mycotoxins in food and animal feed.
In this study, the toxicity of the most well-known and potent carcinogenic mycotoxin, aflatoxin B1 (AFB1), was evaluated with and without RGS treatment using *Artemia salina* larvae and HepG2 human liver cell bioassays. The data obtained confirmed that intact AFB1 is cytotoxic to *Artemia* larvae and HepG2 cells, and it is reduced and significantly detoxified ($p<.0001$, $p<.05$ respectively) after RGS treatment.

Author contributions

Conceptualization: MMN; Formal analysis and investigation: MMN, JFC; Writing – original draft preparation: MMN, JFC; Writing–review and editing: JFC; Validation and visualization: MMN, JFC.

Transparency Statements and Declarations

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Conflict of Interest Statement

The authors declare no competing interests in this study, holding no shares of the company which employed them. The statements and conclusions in this study are based on true observations of the authors.

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