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*Full Length Research Paper*

# Genotoxicity Assessment of Byproducts (Degradants) Resulting from the Treatment of Aflatoxin B1 (AFB1) with NanoGuard's Cold Plasma Technology Using HepG2 Mammalian Cell Immunoblot Analysis and UMU-Chromotest

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## Abstract

**NanoGuard Technologies Inc. has developed a proprietary High Voltage Atmospheric Cold Plasma (HVACP) treatment which degrades Aflatoxin B1 (AFB1) on food, feed, and agricultural products. The technology involves ionization of atmospheric air producing ionized air that contains Reactive Gas Species (RGS). The RGS from air are capable of degrading mycotoxins and the ionized air quickly reverts to air. The objective of this study was to evaluate the genotoxicity of AFB1 before and after NanoGuard's ionized air treatment using immunoblot analysis of histone nuclear protein (H2AX) phosphorylation, and UMU-Chromotest (umu- or umuC test). Nanoguard's ionized air treated AFB1 (test) and untreated AFB1 (control) were used for this genotoxicity evaluation. The HepG2 immunoblot assays showed less induction of γ-H2AX with treated AFB1 compared to the untreated, indicating reduced genotoxicity to human Hep G2 cells. Similarly, UMU analysis with S9 of untreated AFB1 showed genotoxicity as expected, while the treated AFB1 showed no genotoxicity.**

**Keywords:** Mycotoxin genotoxicity assessment, High voltage atmospheric cold plasma (HVACP) treatment, Aflatoxin B1 (AFB1), Histone nuclear protein H2AX phosphorylation (γ-H2AX) immunoblot analysis, UMU (umuC) Chromotest analysis, Ionized air.

## INTRODUCTION

Aflatoxins are a well-studied and characterized group of mycotoxins (Awuchi et al. 2021; El-Sayed et al. 2022; Pankaj et al. 2018) that are known to have detrimental health effects on humans and animals. They are fungal metabolites produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*. Among the four major aflatoxin isomers, AFB1 is the most toxic and most commonly occurring, frequently encountered in cereals, corn, peanuts, cottonseeds, spices, and nuts (Raduly et al. 2020; Sipos et al. 2021). Over the years many methods have been developed to destroy or eliminate aflatoxin in food and feed. Unfortunately, none of them have been quite successful.

Methods such as alkalization, ammonization, and heat or gamma radiation have been explored as post-harvest

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intervention strategies to minimize aflatoxin contamination of different agricultural products (Hochwalt and Keener 2019; Pankaj et al. 2018; Park and Price 2001; Patil et al. 2019). However, these technologies compromise food and feed quality. NanoGuard Technologies has developed an alternative treatment process that reduces mycotoxins and microorganisms on contaminated food and feed (Hochwalt and Keener 2019). The technology utilizes ionized air, also referred to as Reactive Gas Species (RGS), produced using high voltage atmospheric cold plasma (HVACP) dielectric barrier discharge (DBD). Preliminary data have shown that RGS treatment effectively reduces mycotoxin on food and feed without significantly affecting the quality or integrity of the product. Since RGS degrades AFB1 to many products (degradants), the biosafety status of these degradants should be thoroughly studied before any commercial deployment of the technology. The aim of this study was to evaluate the genotoxicity effects of AFB1 degradants resulting from RGS exposure as compared to intact AFB1. We used immunoblot analysis of phosphorylated histone nuclear protein H2AX (γ-H2AX), as well as the umuC test, a scientifically accepted Ames Test method, to determine the genotoxicity effects of intact AFB1 and its degradants from RGS treatment on human cells and bacteria, respectively.

For immunoblot analyses of the accumulated amounts of phosphorylated H2AX (γ-H2AX), cell protein lysates of HepG2 cells were prepared. This cell line has been widely used as a biological model system for studying molecular metabolism, toxicity, and genotoxicity effects of many plants and pharmaceutical based products (Firsanov et al. 2011; Khoury et al. 2020; Quesnot et al. 2016; Tsamou et al. 2012). Moreover, the HepG2 cell line is a key cellular model recommended for determining both cytotoxicity and genotoxicity of environmental factors and pharmaceutical compounds as referenced by Organization for Economic Co-operation and Development (OECD) guideline number 487 (OECD 2016). Studies using the HepG2 cell line have also provided evidence of changes in cell morphology, reductions of cell growth, and eventually cell death as characterized by DNA fragmentation and disintegration resulting from exposure to toxic compounds (Colombowala and Aruna et al. 2022; Motoyama et al. 2018; Podhorecka et al. 2010). Key markers for these destructive processes are caspase activation, aggregation of chromatins, partitions of cytoplasm, and reorganization of nucleus membranes to form bound vesicles (Colombowala and Aruna et al. 2022; Kopp et al. 2019; Toyooka et al. 2017). In addition, the induction and accumulation of phosphorylated histone nuclear protein at Serine 139 (referred as γ-H2AX) in cells has been shown to correlate very well with the amount of DNA damage measured by immunological and immunocytochemical methods (Kopp et al. 2019; Lee et al. 2019; Motoyama et al. 2018; Zhou et al. 2019). This key marker offered a clear way to evaluate mammalian cell DNA disruptions and gauge any genotoxic effects of AFB1 and its degradants produced by RGS. First, the genotoxic effects of various doses of intact AFB1 on the cells were investigated, followed by genotoxicity evaluation of the AFB1 degradants produced by NanoGuard's ionized air treatment relative to untreated AFB1.

For further genotoxicity evaluation, the umuC test was performed. This bioassay utilizes an engineered umu operon of *Salmonella typhimurium* (strain TA1535) which regulates chemical and radiation mutagenesis (genotoxicity). In most organisms, detection and repair of damaged deoxyribonucleic acid (DNA) is achieved through complex signaling pathways involving various DNA damage repair enzymes. In bacteria, this restorative function is generally achieved by the SOS repair system. The SOS repair system includes a promoter containing the DNA damage response genes *recA* and *umuC* (Biran et al. 2010). These genes are ideal targets for genotoxicity evaluations of a wide range of compounds, including water, soil sediment samples, chemicals, food components, cosmetics, and biological fluids (Dizer et al. 2002; Kumar et al. 2021; Mortelmans and Zeiger 2000; Oda 2016).

It is well established that DNA-damaging agents, most mutagens, and carcinogens tend to induce the expression of a umu operon in bacteria cells. Ames and co-investigators demonstrated the success of this approach by engineering bacterial plasmids (pKS1002) fused with the umu operon in a *Salmonella typhimurium* strain TA1535 (Ames et al. 1975). Because of its reliable utility, the engineered bacterium has been validated as an international protocol for the evaluation of genotoxic effects for various environmental and chemical agents (ISO 13829) (Chaudhay and Payasi 2014; Kumar et al. 2021; Oda 2016). In this study, the test was performed with and without S9 activation (S9 fraction from male rat liver). The purpose of including this mixture of enzymes (S9 activation fraction) in the assay was to induce biological transformations of the molecules in question. For more comprehensive evaluations of genotoxic effects of chemicals in bacterial systems, auxiliary metabolic activation agents, such as S9 fraction or Aroclor 1254-induced S9 should be included in the assay (Chaudhary and Payasi 2014; Kumar et al. 2021). The purpose of the addition of such a metabolic activator is to mimic the biological transformations that normally occur in livers which can naturally amplify or diminish the genotoxicity of chemicals. The addition of S9 to this bacterial assay was crucial to thoroughly evaluate the genotoxicity of both AFB1 and its degradants. The assays performed without the addition of S9 were similarly important to ensure that AFB1 was not converted into a more direct-acting genotoxin through RGS treatment.

Generally, harmful biochemical events and DNA-

damaging agents tend to induce the expression of the umu operon, which can then be measured by determining the β-galactosidase activity in the bacterial cells containing the fused *lac* operon coding β-galactosidase enzyme (β-gal), linked with the promoter controlling the expression of umu-related proteins (fusion of *umuC* and *lacZ* genes). The β-gal can then react with an added substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG) to form a yellow-colored compound. This color can be quantitatively measured by a spectrophotometer, providing an indirect measure of the β-gal specific enzyme activity produced by the genotoxic effects of a compound in question (Biran et al. 2010). The color intensity is used as a measurement of DNA damage. This is possible because the enzyme transcription amplification is directly proportional to the level of SOS induction correlated with genotoxic damage to the bacteria. Canada-based company Environmental Bio-Detection Products Inc (EBPI, Burlington, Ontario, Canada) has utilized this bacterial plasmid capability to develop a UMU-Chromotest kit (umuC test) and was contracted by NanoGuard Technologies, Inc to perform this work. According to EBPI's validated protocol, the optical density (OD) reading of the controls and test samples were measured using a spectrophotometer at 600 and 420 nm to calculate the UMU-Induction Ratio (IR). Six dilutions of Umu-Chromotest kit positive standard control [4-Nitroquinoline 1-oxide (4NQO) without S9 and 2-aminoanthracene (2AA) with S9] were included in each test plate to ensure the test's validity. If the positive standard control produced a response greater than 2 (IR>2) then the kit was deemed valid for the assessment of genotoxicity. If the IR of a given dilution of AFB1 or AFB1-degradants was over 1.5 (IR>1.5), then that concentration was considered genotoxic, as per EBPI protocol.

## MATERIALS AND METHODS

### Materials

The 90 mm diameter Whatman™ grade 5, 2.5 µM filter papers were purchased from Amazon.com (Seattle, WA). Purified crystalline AFB1 was purchased from Cayman Chemical Company (Ann Arbor, MI). Methanol and Dimethyl Sulfoxide (DMSO) were purchased from VWR (Radnor, PA). Ultra-purified Milli-Q water was obtained from a Millipore® Advantage A10 system.

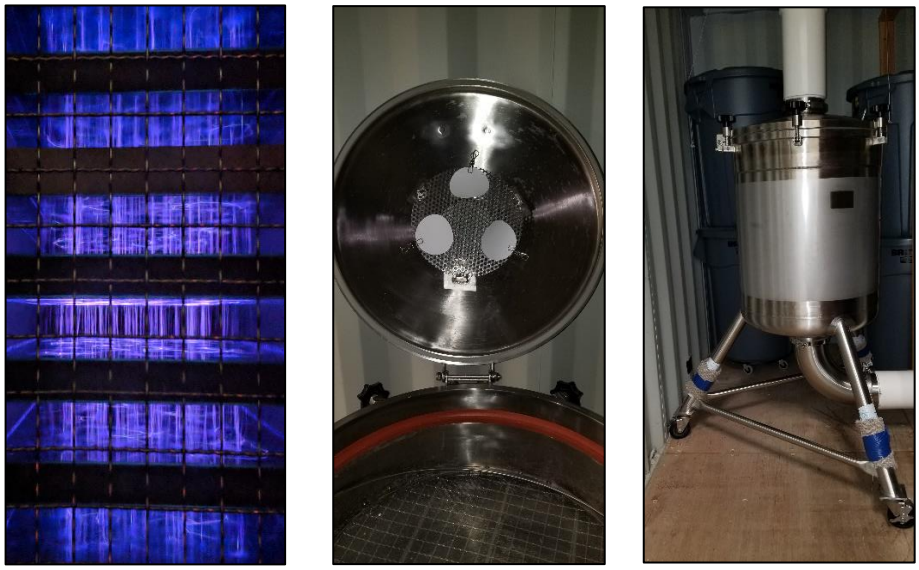
The hepatocyte (HepG2) cell line for genotoxicity studies was purchased from MilliporeSigma (St. Louis, MO). The cell growth medium, Eagle's Minimum Essential Medium (EMEM: 4.5 g/L glucose and L-Glutamine) was bought from American Type Culture Collection (ATCC, Manassas, VA). Medium reagents, streptomycin, penicillin, Fetal Bovine Serum (FBS) and trypsin-EDTA [0.25% trypsin/0.53 mM EDTA in Hank's Balanced Salt Solution (HBSS)] without calcium and magnesium were also purchased from ATCC. The phosphate-buffered saline (PBS) was obtained from CellGro Technologies (Lincoln, NE) and the cell culture flasks and 100 mm petri-dishes were purchased from Midwest Scientific (St. Louis, MO). The electrophoresis apparatus/transfer apparatus were purchased from Thermo Fisher Scientific (Waltham, MA). The Pierce™ BCA protein Assay Kit and Super Signal West Pico Rabbit IgG Detection Kit were also bought from Thermo Fisher Scientific. Sample buffer (2X), protein standard/marker, mini gels (4-20%), cut Whatman™ filter papers for mini gels, nitrocellulose membrane, Coomassie blue stain, Ponceau S stain and Mini-Protean TGY gel were purchased from Bio-Rad Laboratories (Hercules, CA). Primary anti-phospho H2AX monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA), while the secondary antibody and the chemiluminescent detection kit were purchased from Thermo Scientific (Swedesboro, NJ). The imaging system was from Azure Biosystems (Dublin, CA). All the other chemicals and reagents used in genotoxicity assays and Western blot analysis were obtained from MilliporeSigma, VWR International (Radnor, PA), and Thomas Scientific.

For genotoxicity evaluation by umuC test, test materials (AFB1 with and without RGS treatment) were prepared by NanoGuard Technologies, Inc. (St. Louis, MO) and were shipped frozen to EBPI (Canada) overnight. The UMU-Chromotest kit including all the reagents and bacterial strain required for genotoxicity test with and without S9 activation were supplied by the contract laboratory (EBPI, Burlington, Ontario, Canada).

### Methods

#### AFB1 Distribution onto Filter Paper Discs and NanoGuard's Ionized Air Treatment

An AFB1 stock solution was prepared by dissolving 10 mg crystalline AFB1 in 50 mL 100% methanol, followed by vortexing and agitation to fully solubilize the crystals. Then 0.6 mL of AFB1 stock solution was evenly spotted onto each of six 90 mm Whatman™ (Grade 5) filter papers laid out on aluminum foil. The filter papers were allowed to dry in a fume hood for ten minutes before an additional 0.6 ml of the stock solution was added. After drying, the filter papers were randomly sorted into two groups. One set of three papers served as untreated control, and the other set of three papers (test group) was exposed to NanoGuard's ionized air (RGS) treatment for 90 minutes (Figure 1). Figure 1a shows the plasma chamber where the working gas is temporarily ionized to produce RGS. Figure 1b shows the contaminated filter papers clipped in place at the exit (top) of the RGS treatment chamber. Figure 1c shows the sealed contacting chamber, shut during treatment to prevent the escape of the working gas containing RGS. The contactor was approximately 20 meters of pipe-length



A B C

**Figure 1. a)** Cold plasma generator where ionization of working gas (air) occurred, producing RGS (close-up view while operating); **b)** AFB1-contaminated filter paper discs clamped in place in contacting (treatment) chamber; **c)** Sealed contacting chamber during RGS-treatment.

away from the plasma chamber. After treatment, the untreated (control) and RGS-treated (test) filter papers were extracted.

**Extraction of Control and ionized air RGS-Treated Filter Paper Discs**

Each test and control filter paper disc were cut into small pieces, pieces collected in individual tubes and extracted using 6% DMSO-dH2O twice (2x). For the first extraction, 4 mL of 6% DMSO-dH2O was added to each paper-containing tube (3 test and 3 control). The mixture was agitated by alternately shaking and vortexing to facilitate maximum extraction. The tubes were incubated for 5 min at room temperature (RT) and agitated again. The supernatant from each tube was transferred to a clean vial. An additional 4 mL of 6% DMSO-dH2O was added to the paper-containing tubes for a second extraction and the supernatants were pooled together. The recovered supernatants were then centrifuged for 5 minutes at 14,000 x g to settle any remaining paper particles. The total volume of supernatants (extracts) recovered from each filter paper disc ranged from 5.9-6.2 mL. The resulting extracts from each filter paper were then transferred into new tubes, and aliquoted into ten (10) clean vials (10 x 0.6 mL/vial). The 10 vials of each extract were stored in the dark at -20°C until analysis.

**HepG2 Cell Culture**

The HepG2 cell line was chosen for this study because it is derived from the liver, the primary organ affected by AFB1 once ingested by human or animals. To start the HepG2 cell culture, frozen cells in a vial containing 1 mL of freezing medium dimethyl sulfoxide (DMSO) mixture were thawed out and transferred into 15 mL tubes containing 10 mL of fresh serum free EMEM to wash out the DMSO. The cells were centrifuged at 500 x g for 5 min to pellet the cells, and the supernatant containing DMSO was removed and discarded. The cells were then re-suspended in fresh growth medium (EMEM supplemented with 10% FBS, 100 unit/mL penicillin, and 100 µg/mL streptomycin) and transferred into a T-75 tissue culture flask (bottom growth area 75 $cm^2$) containing 15 mL of growth medium. The seeded flasks were incubated at 37°C in a 5% carbon dioxide ($CO_2$) humidified incubator. The cells were allowed to attach to the flask floor and grown to 70% confluence prior to plating for experimentation or passage for culture maintenance. For culture maintenance, the cells were grown as a sub-confluent monolayer in growth medium with media changes every 3-4 days. For passage, the cell monolayer was rinsed once with fresh, warm serum-free medium and 3 mL of warmed 0.25% Trypsin-EDTA solution was added to cover the bottom of the flask. After adding the Trypsin-EDTA, each flask was incubated for 5–7 minutes in the aforementioned $CO_2$ incubator. An equal volume of growth medium was added to the detached cells with gentle resuspension via pipetting to neutralize trypsin activity and prevent additional cell digestion. The cell suspension was split by further diluting in a 1:10 ratio with growth medium, and sub-culturing at 37°C in a humidified incubator with 5% $CO_2$.

**Dose Response Stock Solution Preparation and HepG2 Cell Incubation with AFB1**

AFB1 was dissolved in 6% DMSO-dH2O to prepare a 55 µg/mL solution (stock solution). The concentration of the stock solution was confirmed by High Performance Liquid Chromatography (HPLC) analysis using modified AOAC method #994.08 (Trilogy Analytical Lab, Washington, MO). Appropriate amounts of this stock solution were diluted with serum-free cell medium and used in the AFB1 dose response genotoxicity study. The genotoxicity assay was conducted in 100 mm diameter petri dishes. The final AFB1 concentrations in petri dishes containing 10 mL medium ranged from 0-4.4 µg/mL. Because excessive DMSO negatively impacts the health and metabolism of HepG2 cells, the final DMSO concentration in all AFB1 test petri dishes was maintained at 0.3%, except the 4.4 µg/mL max AFB1 concentration petri dish which contained 0.5% DMSO. The total volume of assay solution in each petri dish was 10 mL.

**AFB1 Dose Response, Cell Lysing and γ-H2AX Immunoblot Analysis**

A range finding dose-response immunoblot assay was done initially to gauge the genotoxicity effects of various concentrations of AFB1 on HepG2 cells. The AFB1 concentrations for this study ranged between 0 and 4.4 µg/mL. Briefly, the HepG2 cells were plated in 100 mm petri dishes at a seeding density of $4x10^6$ cells per dish in 10 mL of medium and grown overnight before exposure to AFB1. The medium in the dish was then removed and replaced with serum free medium containing the appropriate concentrations of AFB1 diluted from the initial working AFB1 stock solution (55 µg/mL in 6% DMSO).

One negative control petri dish was plated which contained only the HepG2 cells in serum free medium with no DMSO, and one vehicle control dish had cells with 0.3% DMSO in serum free medium. Petri dishes (one dish per AFB1 concentration) for each experiment were then incubated for 24-hours in a 5% $CO_2$ incubator before lysing the cells for preparation of protein lysates. Cells were washed once with ice cold phosphate-buffered saline (PBS-1X) and lysed with 1 mL ice cold RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS) for 45 min on ice. The lysed cells were centrifuged at 12,000 x g for 15 min at 4°C for recovery of protein lysates. Protein determination of the recovered lysates was performed using a Pierce™ BCA protein assay Kit (Thermo Fisher Scientific) and the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of phosphorylated histone protein H2AX using specific rabbit monoclonal antibodies as described below. The study was repeated two additional times to ensure the assay was reproducible with consistent results.

**RGS-Treated vs. Untreated AFB1: Comparative γ-H2AX Immunoblot Analysis**

The extraction of both the control and filter paper disc was performed as described above. The resulting supernatants (extracts) from each filter paper were transferred into new tubes, vortexed, and aliquoted into clean vials (0.6 mL/vial) and stored in darkness at -20°C pending lysis for SDS-PAGE and immunoblot analysis as described below.

Briefly, the immunoblotting of the cell lysates (proteins) was performed as previously described (Toyooka et al. 2011, 2017; Zhou et al. 2019). Following protein determination of the recovered lysates using a Pierce™ BCA protein assay Kit (Thermo Fisher Scientific), 40 µg each of test and control lysate (proteins) were loaded into wells of SDS-PAGE mini gels (4-20% gradient). Then electrophoresis of protein-loaded gels was conducted at 100 V in an Invitrogen Mini Protein gel electrophoresis unit. After electrophoresis, the gels were equilibrated in transfer buffer for 15 minutes and packaged for transfer onto nitrocellulose membranes from Thermo Fisher Scientific. The packed nitrocellulose membrane and gel sandwich were placed in the transfer chamber unit with the nitrocellulose paper facing the positive terminal and the gel towards the negative terminal. The transfer was performed at 100 V at room temperature for 1 h. Afterwards, the nitrocellulose membrane blots were blocked with 15 mL of 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h and then probed overnight (at 4°C) for the accumulated γ-H2AX using the primary specific monoclonal antibody for γ-H2AX protein in TBS (1:2000). The membranes were then washed 3x (10 min each) in Tris-buffered saline-Tween 20 (TBST) and incubated with secondary antibody (1:2000) in 10 mL of 5% nonfat dry milk in TBST for 1 h. This was followed by three washes (10 min each) in TBST. The detection of protein bands on the membrane blots was done using Pierce™ Kit ECL reagents according to the supplier's instructions (Thermo Fisher Scientific). The bands' intensities were quantified using an Azure Biosystems C400 Imager and Azurespot Analysis Software (Azure Biosystems, Dublin, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as protein loading control in SDS-PAGE and its band intensities were also measured and used for normalizing γ-H2AX band intensities.

**UMU-Chromotest (Genotoxicity) Analysis**

The UMU-Chromotest (umuC test) assay was performed in accordance with EBPI's standard umuC protocol based upon prior literature (Kwan, K. 1993). EBPI was contracted to perform the umuC test for evaluating the

genotoxicity of NanoGuard's ionized air RGS-treated AFB1 (test) and untreated AFB1 (positive control) filter paper extracts using their commercially available umuC test kit (Product Number 5032). EBPI was not given the sample key in order to eliminate potential bias. Their kit uses the bacterium strain, *Salmonella typhimurium*, containing an engineered SOS promoter that codes for the β-galactosidase (β-gal) enzyme which is linked to the DNA repair gene *umuC.* The test utilized Induction Ratio (IR) values obtained from the assay to determine the genotoxicity of a given solution. Generally, the more color intensity (stronger yellow hue) due to β-gal enzyme activity, the more the genotoxic effect of the compound tested. Further experimental details and the equations used to determine genotoxic activity (Growth Factor, β-galactosidase Activity, and the Induction Ratio) are described in the EBPI standard UMU-Chromotest protocol (TOXI-Chromotest Procedure Manual Version 4.0).

**Statistical Analysis of Data**

The statistical comparisons of the normalized γ-H2AX densitometry units were performed using a 1-tailed, paired t-test of the arcsine-normalized intensity percentages. Prior to statistical analysis, the results were normalized using arcsine normalization. The p-value of p=0.05 was treated as the cut-off point for significant difference. The three untreated AFB1 paper extracts were compared to the three RGS-treated AFB1 paper extract (n=3) after exposing HepG2 cells to the filter paper extracts for 24 hours and analyzing the cell lysate for γ-H2AX and GAPDH (loading protein control) using immunoblot analysis. All error bars and ± signs in all figures represent one standard error from the mean (SEM) in each direction.

The statistical analysis of genotoxicity data from Umu-Chromotest was performed using Microsoft Excel's unpaired, unequal-variance, two-tailed "t-test" function. Prior to statistical analysis, the genotoxicity percentages were normalized using natural logarithmic (ln) normalization, and α=0.05 was set as the significance cut-off point. Both the untreated positive control extracts (n=3) and the RGS-treated paper extracts (n=3) were analyzed by UMU-Chromotest and their genotoxicity (normalized induction ratio) levels were compared by statistical analysis. Calculations of p-values were performed to determine if statistically significant differences between normalized induction ratios were observed as a result of RGS treatment.

**RESULTS**

**AFB1 Concentrations of Extracts**

The AFB1 concentration of each undiluted filter paper extract is summarized in Table 1, as well as the diluted HepG2 and umuC incubation concentrations. The concentrations of the AFB1 stock solution and the six filter paper extracts (in 6% DMSO-dH2O) were determined by HPLC analysis at Trilogy Analytical Laboratory. The data obtained showed minimal variability within each triplicate group of filter paper extracts (RGS-treated and untreated). The extracts from filter papers treated with RGS had minimal amounts of AFB1 compared to extracts from untreated papers (1.9 ± 0.3 AFB1 µg/mL vs. 16.5 ± 1.0 AFB1 µg/mL). On average, this translates to an AFB1 reduction of 88.6 ± 1.7% by treatment with NanoGuard's ionized air/RGS (Table 1).

[1]Starting concentration of each AFB1-spiked filter paper extract

[2]$\text{Recovery \%} = \frac{(\mu\text{g/mL AFB1 recovered in solution}) \times (8\text{ mL 6\% DMSO Extractant volume})}{(201\ \mu\text{g/mL AFB1 in methanol inoculum}) \times (1.2\text{ mL inoculum added to each paper})} \times 100\%$

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

[4]AFB1 concentration in each Untreated (control) or RGS-Treated (test) HepG2 cell plate

[5]Highest AFB1 concentration the *Salmonella typhimurium* were incubated for all six extracts (Serial Dilution 1), followed by two-fold serial dilutions (1:2 dilutions of each extract) up to Serial Dilution 12

[6]Solution of pure toxin prepared by dissolving 10 mg AFB1 with 50 mL methanol.

2 x 0.6 mL aliquots of this solution (241.2 µg) were used to spike each paper with AFB1.

[7]Not applicable.

**HepG2 γ-H2AX Immunoblot Assay Results**

The genotoxicity of intact AFB1 on the HepG2 cell line was evaluated using dose-response immunoblot analysis of induced formation and accumulation of phosphorylated H2AX, also referred to as γ-H2AX (Figure 2). The 0% DMSO negative control lysate (lane 1) showed no phosphorylation while the 0.3% DMSO vehicle control lysate (lane 2) showed a small, basal level of phosphorylation. The exposure of HepG2 cells to AFB1 resulted in notable induction and accumulation of γ-H2AX, with elevated expression as AFB1 concentration increased between 0.2 µg/mL to 4.4 µg/mL. This accumulation was characterized by strong, dark bands representing γ-H2AX (lanes 3-8). The bottom lanes in Figure 2 show the loading protein control (GAPDH) bands for the membranes which were initially probed for γ-H2AX (target protein). The weak GAPDH bands at the edges of membranes are due to washing and stripping the membrane prior to re-probing with the GAPDH antibody, which did not change observed conclusions

**Table 1.** AFB1 Concentrations of Filter Paper Extracts using HPLC.

| Sample Description | Undiluted AFB1 Conc.[1] (µg/mL) | AFB1 Recovery[2] (%) | AFB1 Reduction[3] (%) | HepG2 Assay AFB1 Conc.[4] (µg/mL) | umuC Assay Max In-well AFB1 Conc.[5] (µg/mL) |
|---|---|---|---|---|---|
| AFB1 Stock Solution[6] | 201 | N/A[7] | N/A | N/A | N/A |
| Untreated #1 | 17.9 | 59.2% | N/A | 0.90 | 11.9 |
| Untreated #2 | 17.2 | 56.9% | N/A | 0.86 | 11.5 |
| Untreated #3 | 14.5 | 48.2% | N/A | 0.73 | 9.7 |
| RGS-Treated #1 | 1.4 | 4.7% | 91.4% | 0.07 | 0.9 |
| RGS-Treated #2 | 2.3 | 7.8% | 85.8% | 0.12 | 1.5 |
| RGS-Treated #3 | 1.9 | 6.3% | 88.5% | 0.10 | 1.3 |
| Untreated Extracts Avg ± SEM | 16.5 ± 1.0 | 54.8 ± 3.3% | N/A | 0.82 ± .05 | 11.0 ± .7 |
| RGS-Treated Extracts Avg ± SEM | 1.9 ± 0.3 | 6.3 ± 0.9% | 88.6 ± 1.7% | 0.10 ± .01 | 1.3 ± .2 |

since γ-H2AX densitometry values factored in the GAPDH relative ratio. The results indicate that the exposure of HepG2 cells to AFB1 leads to accumulation of γ-H2AX in a dose dependent manner consistent with previous studies reported in the literature (Quesnot et al. 2016). Thus, AFB1 is genotoxic to HepG2 cells at concentrations of 0.2 µg/mL or greater (Figure 2). This analysis was repeated two additional times with similar results (Gels not shown).

Next, lysates from cells exposed to untreated AFB1 (positive control) extracts were evaluated relative to extracts from RGS-treated AFB1 (test) filter papers. As seen in Figure 3a below, through comparison to the vehicle control lysate (Lane 2), the immunoblot analysis results showed little to no accumulation of γ-H2AX in cell lysates exposed to RGS-treated AFB1 extracts (Lanes 4, 6, 8), greatly different from lysates of the untreated AFB1 extracts (Lanes 3, 5, 7) which showed major accumulation of γ-H2AX. The immunoblot assay was repeated two additional times and in each case produced similar results (Gels not shown).

Quantitative densitometry analyses of γ-H2AX band intensities were performed for the gel (Figure 3a) and results obtained are summarized below (Figure 3b). The densitometry units of untreated vs. RGS-treated AFB1 filter paper extracts were normalized to the background and the loading protein control (GAPDH). The normalized γ-H2AX intensities from the test and positive control extracts were compared to that of the 0.3% DMSO Vehicle Control, and the average relative γ-H2AX induction percentages are plotted below with each error bar representing one standard error from the mean (SEM) above and below the average.

The statistical analysis of data from this study which compared the normalized γ-H2AX densitometry units of RGS-treated and untreated AFB1 extracts showed a p-value of p=0.03, less than the significance cut-off point of p=0.05. Thus, the RGS-treated extracts caused significantly less genotoxic effects than the untreated extracts. Moreover, the RGS-treated extracts induced γ-H2AX levels nearly identical to the amount observed in the 0.3% DMSO Vehicle Control lysate, indicating a lack of genotoxicity to HepG2 cells at an average AFB1/RGS Degradant concentration of 730 ng/mL. Overall, the accu-

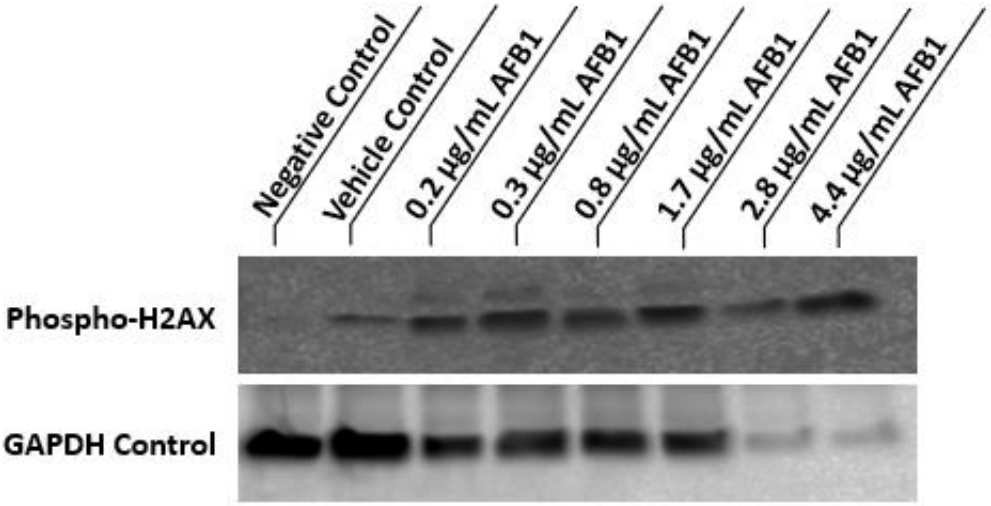



**Figure 2.** Representative immunoblot gel analysis of Phosphorylated H2AX in HepG2 cells exposed to different concentrations of AFB1 for 24 hours. HepG2 cells were exposed to different concentrations of AFB1 in 0.3% DMSO and analyzed using immunoblotting. Protein was separated on 4-20% SDS-polyacrylamide mini gel and blotted with γ-H2AX antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control for protein level normalization.

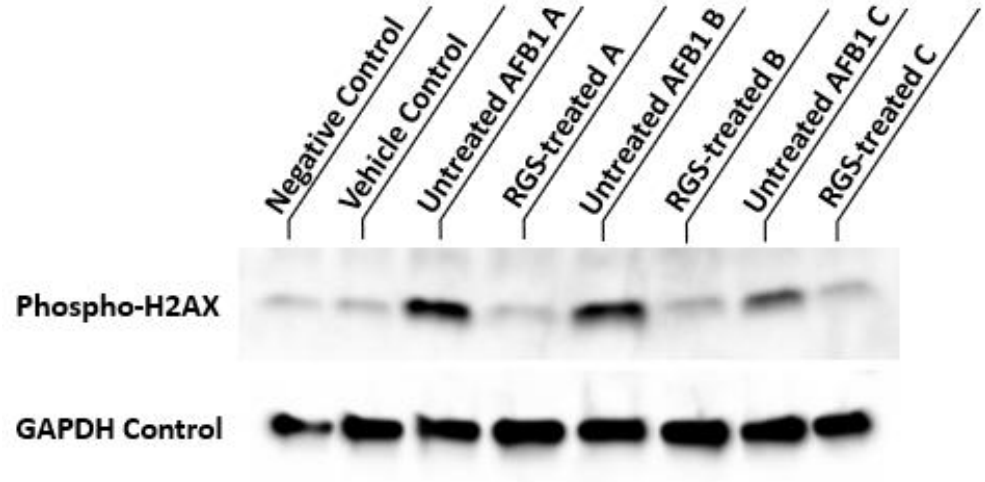



**Figure 3a.** Immunoblot of Phosphorylated H2AX in HepG2 cells exposed to RGS-treated and untreated AFB1 extracts. HepG2 cells were exposed to RGS-treated and untreated AFB1 filter paper extracts for 24 hours. After protein determination, loaded protein was separated on 4-20% SDS-polyacrylamide mini gels and blotted with γ-H2AX antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control for protein level normalization.

mulation of γ-H2AX increased with increasing concentrations of AFB1 in HepG2 cells exposed to AFB1 (Figure 2), and cells exposed to RGS-treated AFB1 showed significantly lower ($p<.05$) expression of γ-H2AX (less genotoxicity) compared to cells exposed to untreated AFB1 (Figure 3a).

**Umu-Chromotest Analysis**

Umu tests were conducted with and without S9 activator at EBPI. The assays were repeated to make sure that the results were reproducible. The results from the first run (Report 1) with and without S9 are summarized in Figures 4 and 5. Each data point represents the average

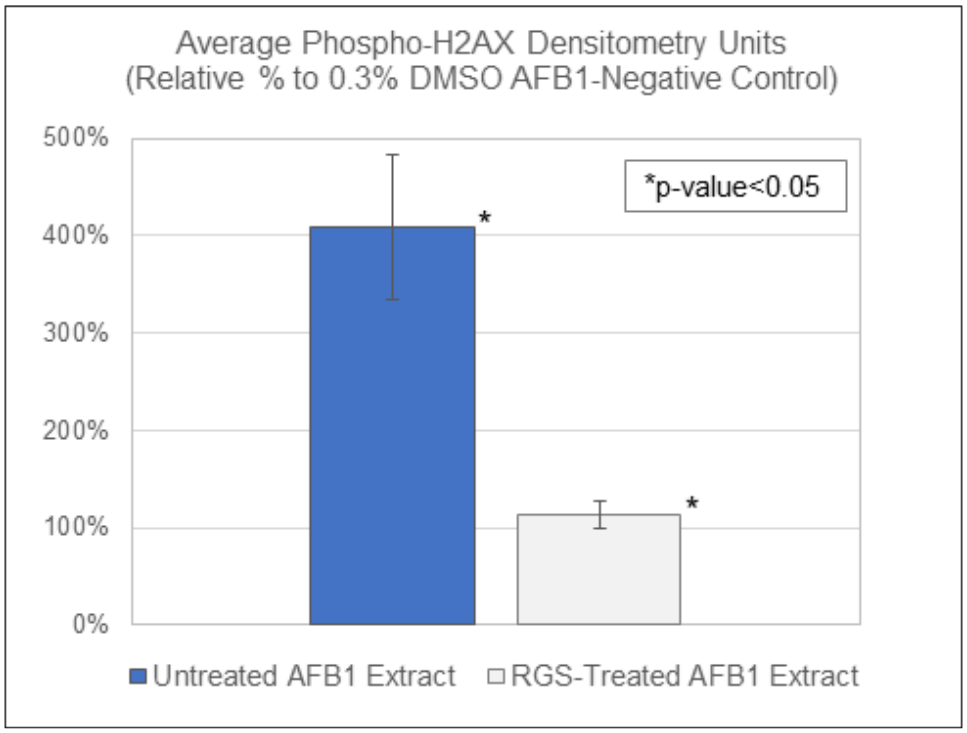



**Figure 3b.** Average Phosphorylated H2AX Percentages of Normalized Densitometry Units Relative to the 0.3% DMSO Vehicle Control. Graph compares γ-H2AX levels in HepG2 cell lysates incubated with RGS-treated AFB1 vs. untreated (positive control) AFB1 extracts (3 data points per group). Values displayed are the average densitometry percentage ratios ± SEM.

IR of the three untreated or RGS-treated AFB1 filter paper extracts at a given serial dilution. The error bar represents one standard error from the mean (SEM) in each direction.

For tests with S9, the RGS-untreated (positive control) extracts produced clear genotoxic effects at the first four dilutions (AFB1 concentrations of 11.0, 5.5, 2.8 and 1.4 µg/mL respectively), while the RGS-treated extracts showed no genotoxicity at all dilutions (Figure 4).

In the absence of S9, both RGS-untreated (control) and RGS-treated (test) extracts showed no genotoxic effects to the bacterial system except at the two highest concentrations (Figure 5).

The lesser observed genotoxic effects without S9 on the bacterial system [*Salmonella typhimurium* (TA1535/pSK1002)] for both untreated and RGS-treated filter paper extracts demonstrated that both control and test extracts have observable but minimal direct genotoxic effects on the *Salmonella typhimurium* testing system. Without S9, the general trend observed was no major differences in genotoxicity between RGS-treated and untreated AFB1.

To confirm the above results, EBPI was contracted to repeat the assay. The assay was performed exactly in the same manner as the first genotoxicity assay using different aliquots of the same extracts, except with two wells per serial dilution (duplicate analysis) instead of just one, making this follow-up analysis slightly more accurate than the first. The IR values of the duplicate analyses of each extract's serial dilutions were averaged (average of two per dilution), then the three duplicate averages were averaged (average of three) for both the RGS-treated and untreated groups to create each statistically analyzed data point. The data are summarized below in Figures 6 and 7, with and without S9, respectively, and the observed trends were the same as the initial study.

As previously seen, extracts with S9 for RGS-treated extracts showed no genotoxicity at nearly all dilutions tested. The singular, minor outlier at RGS-treated serial dilution 3 (IR > 1.5) may be attributed to biological system variance or operator error. On the other hand, the untreated extracts had major genotoxicity for the first eight (8) serial dilutions corresponding to AFB1 concentrations ranging from 11,000 – 86 ng/mL. Statistically significantly less genotoxicity was observed from the RGS-treated extracts than the untreated extracts at serial dilutions 1-8 (Figure 6).

Regarding the data without S9, the assay once again showed a lack of genotoxic effects for both control and test extracts except at the first two serial dilutions. No statistically significant differences between test and control sample genotoxicity were observed at any dilution (Figure 7).

Overall, the results showed that both untreated and RGS-

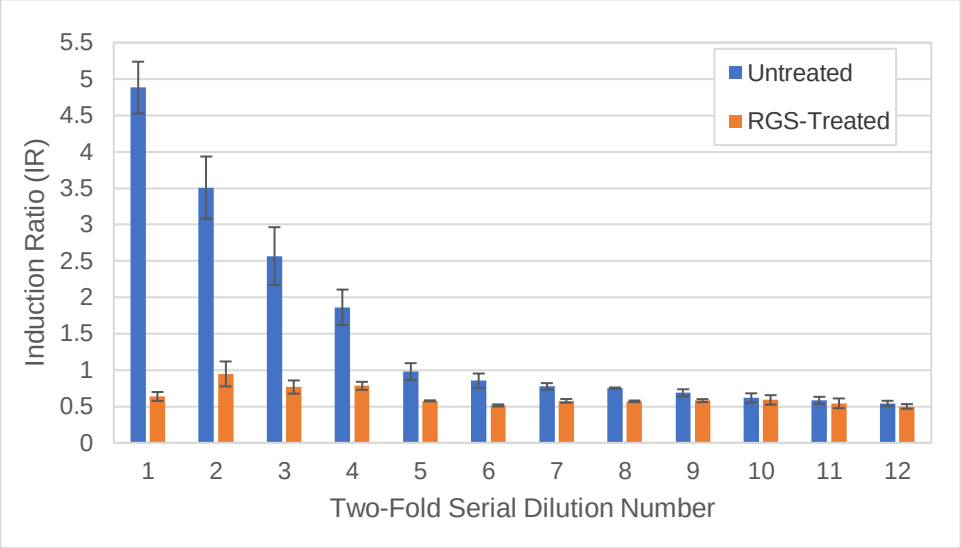



**Figure 4.** UMU-Chromotest Assay Initial Results with S9 Activation.

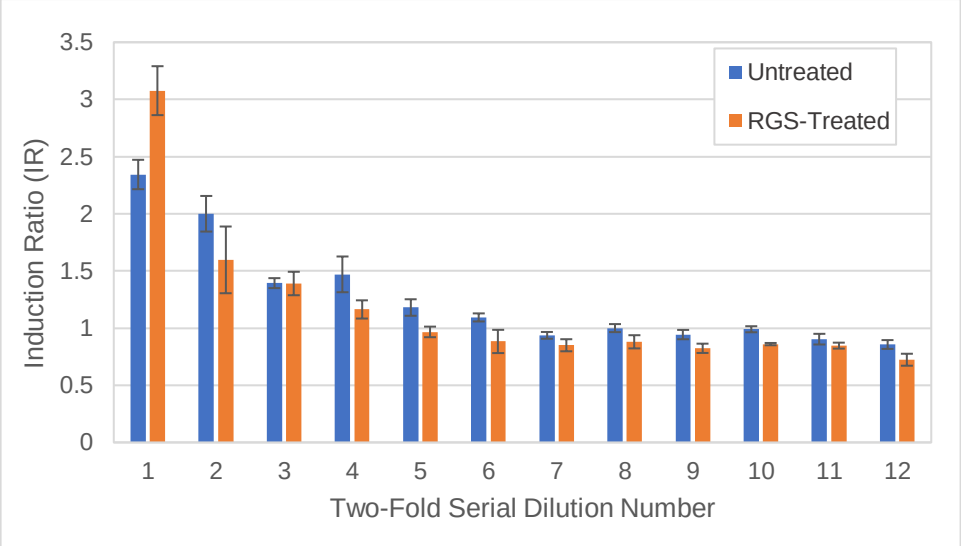



**Figure 5.** UMU-Chromotest Assay Initial Results without S9 Activation.

treated AFB1 cause minor direct genotoxic effects (without S9) to the bacterial system at high concentrations, while metabolic activation by S9 caused major genotoxicity only in the untreated AFB1 extracts. The follow-up assay results demonstrated the reproducibility of the assay, reinforcing the observation that NanoGuard's RGS treatment greatly reduces the genotoxicity of AFB1 when liver enzyme is present.

## DISCUSSION

The potent, naturally occurring carcinogen Aflatoxin B1 presents a danger to food supplies all around the world. NanoGuard Technologies' ionized air or RGS treatment reduces the quantity and concentration of this dangerous compound through conversion to degradants of differing molecular structures. Although a reduction of Aflatoxin B1 improves the safety of any contaminated food, the potential genotoxic effects of NanoGuard's RGS-produced AFB1 degradants have not been thoroughly investigated. It was therefore imperative to study the genotoxicity of these AFB1 degradants.

In the present study, we investigated the genotoxicity effects of AFB1 and its breakdown products from ionized air/RGS treatment using two *in vitro* assays. This was done by quantifying HepG2 cell expression and accumulation of γ-H2AX protein, as well as UMU-Chromotest analysis.

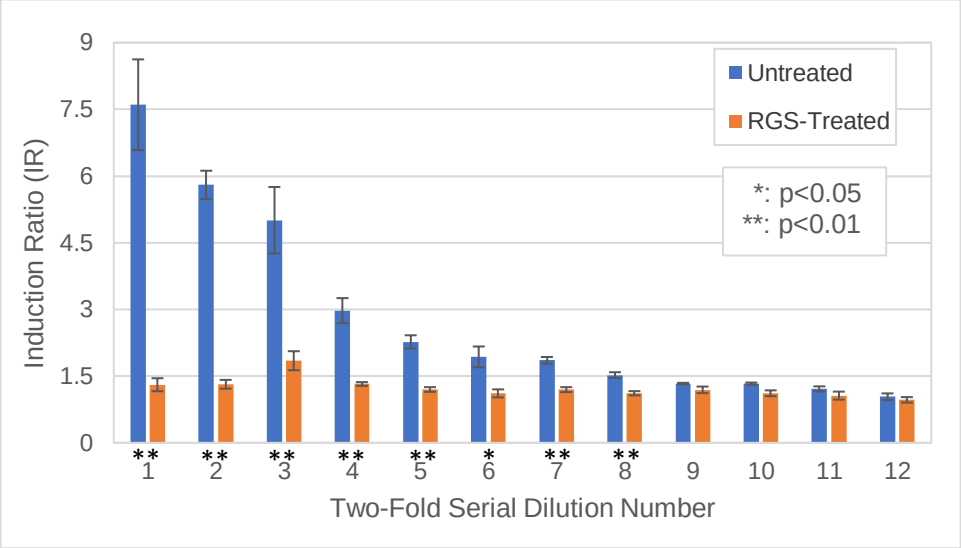



**Figure 6.** UMU-Chromotest Assay Repeated Results with S9 Activation.

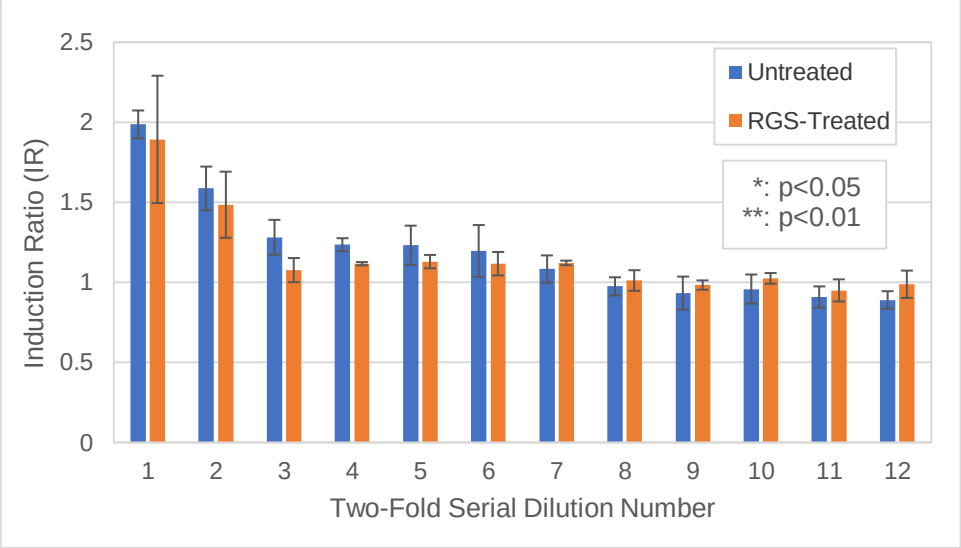



**Figure 7.** UMU-Chromotest Assay Repeated Results without S9 Activation.

First, immunoblot analysis using HepG2 cells was used to determine the level of phosphorylation of H2AX in the cells. This technique has been used many times to measure genotoxicity in various cell types (Firsanov et al. 2011; Kopp et al. 2019; Lee et al. 2019; Motoyama et al. 2018; Toyooka et al. 2011). It has an advantage over other DNA damage markers because of its high specificity, sensitivity, and also the ability to detect small changes of genome integrity in cells (Firsanov et al. 2011; Khoury et al. 2020; Lee et al. 2019; Motoyama et al. 2018; Toyooka et al. 2017). It was expected that the well-known potent mycotoxin, AFB1, would initiate DNA damage and activate the DNA-damage response (DDR) mechanism, leading to induction and accumulation of the phosphorylated form of H2AX (γ-H2AX). Phosphorylation of H2AX is the first signaling mechanism for DNA damage and repair in cells, known to be caused by chromosomal disruptions like DNA fragmentation or Double Stranded Break (DSB) (Firsanov et al. 2011; Podhorecka et al. 2010; Stenvall et al. 2020). It is also known that genotoxic compounds tend to induce cellular DNA damage, thus priming the cells for apoptosis and cell disintegration (Khoury et al. 2020; Podhorecka et al. 2010; Zhou et al. 2019). This knowledge has led to the

use of this *in vitro* tool for determination of DNA disruption and genotoxicity as a result of physical, chemical, environmental, and pharmaceutical compounds towards mammalian cells. Several independent studies have reported that exposure of mammalian cells to toxic reagents disrupts chromosomal structure and causes DNA damage in various cell models (Firsanov et al. 2011; Quesnot et al. 2016; Khoury et al. 2020). In contrast, nontoxic exogenous reagents do not cause DNA damage or strand breaks, resulting in no cellular accumulation of γ-H2AX.

The immunoblot dose response assay results obtained showed γ-H2AX induction increased with AFB1 concentration in HepG2 cells (Figure 2). The comparative assessment between RGS-treated and untreated AFB1 extracts showed that NanoGuard's RGS treatment significantly ($p<.05$) reduced the genotoxicity of the AFB1 as measured by GAPDH-normalized γ-H2AX densitometry (Figures 3a and 3b). Thus, it was shown that NanoGuard Technologies' ionized air or RGS treatment neutralizes mycotoxin genotoxicity and mitigates AFB1's apparent harmful DNA-damaging effects to humans and animals. NanoGuard wanted this observation confirmed using an alternative genotoxicity-analysis system performed by a third-party toxicity-screening laboratory. The treated and untreated AFB1 extracts were thus analyzed using Umu-Chromotest at EBPI.

The UMU-Chromotest using bacterium *Salmonella typhimurium* (TA1535/pSK1002 strain) is a well-established tool for evaluating DNA-damaging potential of a variety of compounds, including AFB1 and its breakdown products (Kumar et al. 2021; Oda 2016; Zhou et al. 2019). The induction of umu operon is measured by colorimetric determination of the β-gal enzyme activity following incubation with the test compounds. Generally, genotoxic materials cause DNA damage and activation of the promoter SOS DNA. This promoter carries the repair gene and the *β-gal* gene which in turn activates the transcription of the β-gal enzyme. The activation cascade leads to proportional expression of the *β-gal* gene and the formation of the β-gal enzyme. As a result, the expressed β-gal enzyme produces a color change which is relatively proportional to the level of genotoxic damage to bacterial DNA when chromogenic substrate (ONPG) is added. This scientifically accepted genotoxicity method was used to evaluate the biosafety status of AFB1, and the AFB1/RGS degradant products produced by NanoGuard's ionized air treatment.

Aliquots of the AFB1 extracts (positive control) and RGS-treated AFB1 extracts (test) used in the HepG2 assays were also used for the umuC study. All solutions were analyzed for genotoxicity by EBPI, including the 6% DMSO in dH2O extractant (vehicle control) which was determined to be nontoxic. 2-Aminoanthracene (2AA) and 4-Nitroquinoline (4NQO) have been determined to show strong genotoxic effects on *Salmonella typhimurium* bacterium system and these solutions were thus used as positive standard assay validation solutions for the UMU-Chromotest in accordance with EBPI's protocol. In a valid umu test, these established standard validation solutions produce an Induction Ratio (IR) response greater than 2 (IR>2), and EBPI's analyses were deemed to be valid by their definition. Furthermore, EBPI protocol defined a positive genotoxicity response for a solution as one which produced an IR value greater than 1.5 (IR>1.5).

The umu test results from EBPI are summarized in Figures 4-7. The data show that without S9, both untreated AFB1 and AFB1 treated with NanoGuard's ionized air had similar direct-acting effects on the *Salmonella typhimurium* bacterium system, marked by minimal genotoxic effects at the two most concentrated dilutions for the two extracts (Figures 5 and 7). However, for comprehensive genotoxicity analysis more relevant to higher organisms like mammals, there is a need to include an auxiliary metabolic activation agent to the bacteria, like the S9 fraction or Aroclor 1254-induced S9 (Kumar et al. 2021; Mortelmans and Zeiger 2000). Biological activation of many toxic compounds normally occurs in the liver. Addition of S9 from rat liver in the assay thus mimics this natural biological activation.

With S9, the ionized air treated AFB1 extracts showed no genotoxicity at all tested dilutions while the RGS-untreated AFB1 extracts were very genotoxic at dilutions 1-4 and moderately so at dilutions 5-8 (Figures 4 and 6). The results with S9 clearly show that NanoGuard's gaseous treatment reduces AFB1 genotoxicity. However, the assays performed without S9 were still very important because they evaluated if RGS treatment converts AFB1 into a more direct-acting genotoxin. Because no significant difference was observed between untreated and RGS-treated AFB1 without S9 activation, it was concluded that AFB1 is not converted into a more direct-acting genotoxin.

## CONCLUSION

The level of accumulation of γ-H2AX in HepG2 cells is a reliable marker for measuring deoxyribonucleic acid (DNA) damage caused by a substance. The Umu-Chromotest using *Salmonella typhimurium* (TA1535/pSK1002 strain) is another reliable *in vitro* genotoxicity testing protocol. In this study, we used these two well established *in vitro* genotoxicity assays to investigate whether NanoGuard's ionized air or RGS treatment reduces AFB1 genotoxicity. Both methods showed that RGS treatment significantly reduces the genotoxicity of AFB1. Both methods also showed that AFB1 is a potent genotoxin while the AFB1/RGS degradation products are not. These observations are in alignment with the observed reduction of AFB1 cytotoxicity after ionized air treatment using parallel

toxicological studies performed using *Artemia salina* bioassays as well as MTT assays using the same HepG2 cell line (article under review).

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