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# EFFECT OF AFLATOXIN B1 ON THE HAEMATOLOGY, TESTES, AND LUNGS OF ADULT MALE RATS: A PHYSIOLOGICAL AND HISTOLOGICAL STUDY

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**SUMMARY**

**Aflatoxins are food-contaminating metabolites that can intoxicate and damage multiple organs. This study compared the effects of administration OF aflatoxin powder (AFP) and aflatoxin liquid (AFS) on the testes and lungs of rats and explored their potential mechanisms of action.**

**Fifteen rats were divided equally into 3 groups. Aflatoxin B1 powder (0.5 mg/kg) and aflatoxin B1 liquid (0.5 mg/kg) were orally administered to the AFP and AFS group, respectively, for 21 days. The control group was fed a basal pellet diet and water. The rats were weighed at the beginning of the trial and on the 7th, 14th, and 21st days. Blood samples were drawn at the end of the experiments and analysed for complete blood count (CBC), i.e. red blood cells (RBCs), haemoglobin content, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white**

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**blood cells (WBCs), and platelet, neutrophil, monocyte, lymphocyte, basophil, and eosinophil counts. The levels of malondialdehyde (MDA) and nitric oxide (NO) as oxidative stress biomarkers were determined, as well as serum testosterone. Testes and lungs were examined under a light microscope to assess the aflatoxin B1-induced histopathological changes.**

**Weight gain and its percentages were significantly higher in the AFS group than in the AFP group ($P$ <0.050). Neutrophil, lymphocyte, monocyte, and basophil counts were also significantly increased in the AFP and AFS groups in comparison to the controls. A significant increase in eosinophil count was noted in the AFP group as compared to controls, while the lymphocyte count was significantly higher in the AFS group than in the AFP group. Testosterone serum levels significantly decreased in both groups (AFP and AFS) relative to the control group. MDA and NO serum levels were also significantly higher in the AFS group than in the AFP group, while the levels in both groups (AFP and AFS) were higher than in controls. Histological examination of the testes revealed severe testicular damage, the loss of the spermatogenesis process, and necrosis, degeneration, shrinkage, and atrophy of the seminiferous tubules in both the AFP and AFS groups. Histological observations of the lungs showed large numbers of inflammatory cells, mast cell infiltration, dilated alveolar sacs with exudates, and marked haemorrhage in both groups (AFP and AFS). The histological damage to the testes and lungs was more prominent in the AFS group than in the AFP group.**

**The results demonstrated elevated oxidative stress markers and reduced serum testosterone levels in AFP and AFS-treated rats. Both organs studied (testes and lung) were severely damaged, with more pronounced changes in the AFS-treated group than in the AFP group.**

**KEY WORDS: Aflatoxin B1, Lung, Oxidative stress, Rats, Testes.**

**Introduction**

Global awareness of mycotoxin toxicity to humans and animals has grown considerably in recent years. Aflatoxins are highly toxic mycotoxins produced by *Aspergillus flavus and Aspergillus parasiticus* during the production, harvesting, processing, and storage of agricultural products. Airborne particulate matter containing aflatoxins can be released into the air during the storage and processing of contaminated cereals, increasing the risk of aflatoxin exposure for animals and humans. Consumption of contaminated feed by ruminants and poultry may also lead to the contamination of milk, meat, and eggs (Rushing and Selim, 2019). Aflatoxins are of different types. *Aspergillus flavus* produces aflatoxins B1 and B2, while *A. parasiticus* produces aflatoxins G1, G2, B1, and B2 (Mtimet et al., 2015).

Aflatoxin B1 (AFB1) is the most prevalent and hazardous mycotoxin, associated with growth retardation, mutagenesis, and carcinogenesis (Dai et al., 2017). Genotoxic AFB1 (Klvana and Bren, 2019) can impair the functioning of the liver (Udomkun et al., 2017), kidneys (Wang et al., 2018), heart (Mohamed and Metwally, 2009), testes (Supriya et al., 2014), lungs (Lee et al., 2016), and spleen (Xu et al., 2019). The purity of aflatoxin in foods determines its ability to target multiple organs. For example, pure aflatoxin can affect the heart, lungs, and kidneys while damaging the liver

as the main target (Karmanov et al., 2021). AFB1 is also associated with male infertility through testes impairment (Supriya et al., 2014). Male infertility refers to problems associated with the male reproductive system (spermatogenesis) (Kilchevsky and Honig, 2012). Multiple studies have confirmed an AFB1-induced reduction in sperm quality and quantity leading to male infertility (Supriya et al., 2014). However, the mechanisms associated with infertility are not completely understood. Spermatogenesis and male reproductive health require a stable testicular microenvironment (Yi et al., 2018). The blood-testes barrier (BTB) between neighbouring Sertoli cells is composed of tight junctions (TJ), gap junctions (GJ), and basal ectoplasmic specializations (ES). BTB is a physical barrier in the testes that provides a stable environment for spermatogenesis and restricts the entry of harmful compounds into the seminiferous tubules to prevent the disruption of spermatogenesis and the production of abnormal sperm (Mruk and Cheng, 2015). Spermatocyte differentiation and meiosis for sperm production depend on BTB integrity. A toxin can damage the BTB and induce reproductive toxicity (Cao et al., 2020).

Aflatoxin damage to the respiratory system has been reported in various studies (Lee et al., 2016; Massey et al., 2000; Larsson and Tjälve, 2000). Food industry employees are more exposed to AFB1 and may inhale dust tainted with the toxin during grain processing. The nasal mucosa may further transform the aflatoxins into active metabolites (Larsson and Tjälve, 2000). The capacity of the lungs for glutathione-S-transferase-based AFB1 detoxification is limited, which explains their susceptibility to AFB1 damage (Coulombe Jr, 1994). The present study compared the toxic effects of 21-day administration of aflatoxin powder (AFP) and aflatoxin liquid (AFS) on the testes and lungs of male rats and explored their potential mechanisms.

## MATERIAL AND METHODS

### *Fungal isolate*

Aflatoxin-producing reference strain *Aspergillus flavus* CYA (AUMC 9779) was obtained from Egypt (courtesy of Professor Ahmed Y. Abdelmalek, Moubasher Mycological Center, Assiut University, Assiut, Egypt). The isolate was cultured on Potato Dextrose agar (PDA) (HiMedia, Mumbai, India) slants.

### *Isolate maintenance and storage*

To preserve and store the *A. flavus* isolate for a longer period, it was cultured on Sabouraud Dextrose Agar (SDA) (HiMedia) plates, which were incubated for 5–7 days at 25±2°C. A 15% sterile glycerol solution was used to inoculate *A. flavus* colonies (grown on SDA) in sterile microfuge tubes (250 µl) to preserve and maintain the vitality of the fungal spores at −80°C (Nielsen and Smedsgaard, 2003).

### *Preparation of broth media*

Sabouraud dextrose broth (SDB) (HiMedia) was prepared according to the manufacturer's instructions. Media aliquots (150 ml) were autoclaved for 15 min at 121°C and 1.5 atmospheric pressure in 250 ml flasks. Then the flasks were kept refrigerated until use.

### *Production, extraction, and determination of aflatoxin from* **Aspergillus flavus**

Aflatoxin production was carried out by cultivating (5–14 days) *Aspergillus flavus* in SDB under shaking in aerobic conditions at 25±2°C. Then the culture was filtrated through sterile filter papers

(MN 615, ∅ 150 mm) (Macherey-Nagel, Duren, Germany) using a sterile funnel and flask. Conical tubes (15 ml) (Plastilab, Roumieh, Lebanon) were used to collect the filtrates. Filtrates were stored overnight at a cryogenic temperature (−80°C) to weaken the fungal cell walls, which facilitates solvent penetration into the cell for the extraction of secondary products (Schmitz et al., 2010). Then the filtrates were transferred to a hydrophilic bottle containing methanol (15 ml) (Merck KGaA, Darmstadt, Germany) and again left for 30 min under ultrasonic vibration (Nielsen and Smedsgaard, 2003). The hydrophilic bottle was placed on an orbital shaker (100 rpm) for 30 min, and this step was repeated multiple times. The extract was filtered through micropore filter paper (Bedford, MA, USA) connected to a sterile glass funnel and concentrated by evaporation under a nitrogen flow. Finally, the extract was dissolved in methanol (1.5 ml) (Merck KGaA) and analysed by GC (Shimadzu, Kyoto, Japan) and HPLC (Shimadzu) (Bertrand et al., 2013). The extracted toxin was compared to a standard extract of crude aflatoxin B1 (courtesy of Prof. Ahmed Abdelmalek of the Moubasher Mycological Center, Assiut University, Assiut, Egypt).

***Detection of aflatoxin production***

Aflatoxin production was quickly detected using the ammonia vapour method, which turns toxin-secreting colonies pink on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) culture plates. Ammonia solution (25%) (Merck KGaA) was prepared by adding 25 ml of ammonia to 75 ml of distilled water. A 0.2 ml volume of ammonia solution was placed on the inner side of the Petri dish cover followed by incubation at 25°C for 24 hours, which caused a red colour to appear at the bottom the of fungal colonies (Saito and Machida, 1999).

***Animals***

Fifteen adult male Wistar albino rats (7–8 weeks old) weighing between 180 and 200 g were obtained from the College of Pharmacy, Umm Al-Qura University, Makkah, KSA. The rats were kept in clean sterile polypropylene cages and exposed to a normal 12-hour light/dark cycle throughout the experimental period (21 days). The cages with experimental rats were placed in air-conditioned rooms (60–65% humidity and 21–23°C) for 2 weeks before the start of the experiments. They were fed a basic diet and water to ensure their behaviour, growth, and adaptation to experimental conditions.

***Experimental design***

The rats (15) were divided equally and randomly into 3 groups (5 rats in each group). The control group was fed water and basal pellet diet. The AFP group received aflatoxin B1 powder (0.5 mg/kg of body weight) orally for 21 days and was fed a basal diet. The AFS group received aflatoxin B1 liquid through a gastric tube (0.5 mg/kg of body weight) for 21 days and was fed a basal diet (Yilmaz et al., 2018).

***Biological tests***

The rats were weighed at the start of the experiment and on the 7th, 14th, and 21st days. Weight gain (final body weight – initial body weight) and weight gain percentage (weight gain / initial body weight × 100) were calculated.

***Biochemical study***

The rats were sacrificed at the end of the experimental period, and blood samples were collected by cardiac puncture. The blood of each animal was split into two types of blood sampling tubes: a plain tube for biochemical assays and a tube containing tri-potassium ethylenediaminetetraacetic acid (K3-EDTA) for haematological assays. Blood samples were added to the EDTA tube to retrieve plasma for the complete blood count (CBC), i.e. red blood cells (RBCs), haemoglobin content, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBCs), and platelet, neutrophil, basophil, lymphocyte, eosinophil, and monocyte counts. Serum extraction was performed by collecting the blood in a test tube without anticoagulant followed by centrifugation (3000 rpm) for 15 minutes. Then, the serum aliquot was stored at −80°C for further use in biochemical and hormonal analyses. Rat-specific ELISA kits (Bender MedSystems Gmbh, Wien, Austria) were used to determine oxidative stress biomarkers (malondialdehyde (MDA) and nitric oxide (NO)) and serum testosterone levels.

***Histological study***

The rats were killed by cervical dislocation at the end of the experimental period (21 days). Testes and lungs were immediately isolated, rinsed with water to remove blood, and kept in formalin (10%) at room temperature for 24 hours. The specimens were taken out of the buffered formalin, processed through a graded series of ethanol and xylene (Merck KGaA), embedded in paraffin, sectioned (5 µm thickness) using a rotary microtome, slide-mounted, and stained with haematoxylin-eosin (Carleton, 1980). The slides were examined under a light microscope (Olympus) (Evident, Tokyo, Japan) for aflatoxin B1-induced histopathological changes in the organs of the male Wistar rats.

***High-performance liquid chromatography (HPLC) of aflatoxin B1***

Aflatoxin B1 ($C_{17}$ $H_{12}$ $O_6$) is a colourless to pale yellow crystal or white powder with a molecular weight of 312.27. The HPLC system was a Beckman system (goldTM) with solvent modules (127 pumps) and a programmable UV detector. The isocratic mobile chromatography phase comprised $H_2O$ (80%) and MeOH (20%) (v/v). A guard column-protected 5-µm reverse phase column (LiChrosphere® C18; 250 mm x 4 mm non-polar) filtered the mobile phase and pumped it to waste (1 ml/min). The wavelength was set to 375 nm, and the system had an injection loop of 10 µl. Pressure (50–350 bars) was applied frequently to push the liquid mobile phase through the column with a solid stationary phase. Freshly prepared aflatoxin B1 samples (100 µl) were injected into the system. Prior to sample injection, pumps were primed and methanol was used for the first run to clean the injection loop and column of impurities.

***Gas chromatography (GC) analysis***

Gas chromatography uses gas as the mobile phase and liquid confined to solid particles (stationary phase). During GC analysis, the polyphase (diphenyl 35% / dimethylsiloxane 65%) is required to withstand the effects of the heating and silylating agents. A fast-temperature program was applied for aflatoxin B1 separation. Initial and final temperatures were set to 50°C and 250°C, respectively, with a carrier gas flow rate of 2 ml/min and a heating rate of 15°C/min. UV absorbance of aflatoxin B1 was monitored at a wavelength of 272 nm. GC analysis was carried out in a Shimadzu model GC15 (Japan) fitted with the Shimadzu Chromatopac C-R4A chromatogram integrator. Sample injection was carried out using an OC-9 capillary on a column injector. A fused silica

capillary column with chemically bonded phenylmethyl silicon liquid phase (5%) (DB-5, 0.25 mm i.d., J & W, CA, USA) was used for the GC analysis.

***Statistical analysis***

SPSS Statistics (IBM SPSS, N.Y., USA) was used for the statistical analysis. Normal data distribution was evaluated using the Shapiro–Wilk test. One-way ANOVA with Tukey's LSD test assessed the significance among treatment groups at a *P*-value of <0.05. Data were expressed as mean ± standard error.

## RESULTS

***Body weight***

Table 1 presents the effects of the AFP and AFS treatments on the rats' body weight after 7, 14, and 21 days. Insignificant differences were observed in the body weight of the groups at different intervals, while weight gain and weight gain percentages were significantly higher in the AFS group than in the AFP group ($P$ <0.050).

**Table 1.**
Effects of AFP and AFS treatments on rats' body weight after 7, 14, and 21 days

| Parameters | Control | AFP | AFS |
|---|---|---|---|
| **Body weight on day 1 (grams)** | 265.20±2.13 | 270.60±0.40 | 266.00±2.45 |
| **Body weight on day 7 (grams)** | 278.20±2.01 | 278.00±5.83 | 276.00±4.00 |
| **Body weight on day 14 (grams)** | 285.60±3.01 | 286.00±6.00 | 284.00±4.30 |
| **Body weight on day 21 (grams)** | 287.20±3.48 | 284.00±.290 | 290.80±4.41 |
| **Body weight gain (grams)** | 22.00±2.51 | 14.00±2.90 | 24.80±2.80[a] |
| **Body weight gain percentage (%)** | 8.29±0.93 | 5.19±1.07 | 9.31±1.01[a] |

Values in the same row are significantly different; a – significance vs AFP ($P$ <0.050).

***Haematological outcomes***

The RBC count, haemoglobin content, MCV, MCH, and platelet count of the control, AFB, and AFS groups were insignificantly different ($P$ >0.05). However, the neutrophil, lymphocyte, monocyte, and basophil counts were significantly higher in both groups (AFP and AFS, $P$ <0.050) than in the controls. A significant increase in the eosinophil count was noted in the AFP group relative to controls ($P$ <0.050), while the lymphocyte count was significantly higher in the AFS group than in the AFP group ($P$ <0.050) (Table 2).

**Table 2.**
Effect of AFP and AFS administration on complete blood count (CBC) of rats after 21 days of treatment

| Parameter | Control | AFP | AFS |
|---|---|---|---|
| **RBCs** ($X10^6$/UL**)** | 8.70±0.30 | 8.59±0.06 | 8.34±0.11 |
| **Haemoglobin** (g/dl) | 16.10±0.03 | 16.44±0.29 | 16.16±0.17 |
| **MCV** (fL) | 52.32±1.26 | 52.72±0.70 | 54.04±0.83 |
| **MCH** (pg/dl) | 18.76±0.60 | 18.94±0.13 | 19.40±0.11 |
| **MCHC** (g/dl) | 35.84±0.50 | 35.94±0.30 | 34.68±0.36 |
| **Platelets** ($X10^3$/UL**)** | 710.40±83.66 | 818.60±33.93 | 804.80±70.05 |
| **WBCs** ($X10^3$/UL**)** | 11.92±0.86 | 11.00±1.40 | 10.86±1.57 |
| **Neutrophil count** ($X10^3$/UL**)** | 0.62±0.22 | 6.10±1.57$^{b*}$ | 5.30±1.09$^{b*}$ |
| **Lymphocyte count** ($X10^3$/UL**)** | 11.28±0.73 | 55.04±1.24$^{b***}$ | 66.74±4.02$^{b***}$ /a* |
| **Monocyte count** ($X10^3$/UL**)** | 0.44±0.06 | 21.88±1.11$^{b***}$ | 22.10±0.56$^{b***}$ |
| **Eosinophil count** ($X10^3$/UL**)** | 0.64±0.27 | 1.42±0.10$^{b*}$ | 1.16±0.15 |
| **Basophil count** ($X10^3$/UL**)** | 0.37±0.01 | 1.98±0.21$^{b***}$ | 1.84±0.10$^{b***}$ |

Values in the same row are significantly different; b – significance vs control; a – significance vs AFP. $^{*}P$ <0.050; $^{***}P$ <0.001.

***Oxidative stress markers***

A significant rise in MDA and NO serum levels was noted in the AFP and AFS-treated groups in comparison to controls ($P$ <0.0001). These levels (MDA and NO) were also significantly higher in the AFS group than in the AFP group ($P$ <0.050 and $P$ <0.010, respectively) (Table 3).

**Table 3.**
MDA and NO serum levels after 21 days of AFP and AFS administration

| Parameter | Control | AFP | AFS |
|---|---|---|---|
| **MDA** (Miu/L**)** | 0.30±0.02 | 1.55±0.07$^{b***}$ | 1.95±0.12$^{b***/a*}$ |
| **NO** (Mico/L**)** | 27.20±0.49 | 75.60±2.50$^{b***}$ | 92.20±3.57$^{b***}$ /a** |

Values in the same row are significantly different; b – significance vs control; a – significance vs AFP. $^{*}P$ <0.050, $^{**}P$ <0.010, $^{***}P$ <0.001.

***Serum testosterone level***

Serum testosterone levels significantly decreased in the AFP (14.86±0.45 nmol/L, $P$ <0.050) and AFS (13.32±0.69 nmol/L, $P$ <0.010) groups relative to controls (17.88±0.93 nmol/L) (Fig. 1).

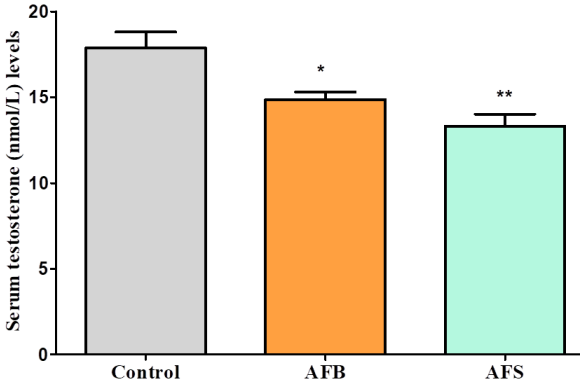


**Figure 1.** Serum testosterone levels after 21 days of AFP and AFS treatments. Significance vs control: *$P$ <0.050; **$P$ <0.010.

***Histological impact of AFP and AFS on testes***

The testes of the control rats presented a normal histological structure of the seminiferous tubules with complete spermatogenesis and normal series of Sertoli cells and associated spermatogenic cells (Figure 2A). The seminiferous tubules appeared as round or oval structures. They were surrounded by a thin basement membrane and contained several cellular layers, including spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa. The intratubular tissue formed

of connective tissue holds the seminiferous tubules together and contains blood vessels. It also contains testosterone- secreting Leydig interstitial cells (Figure 2B).

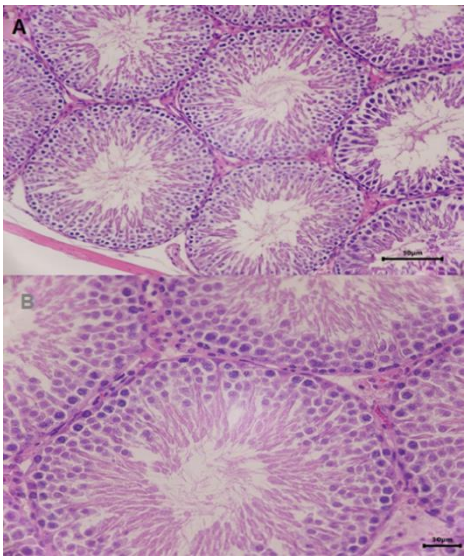


**Figure 2.** Microscopy of control rat testis presenting (**A**) normal seminiferous tubules with a series of spermatogenic cells (arrow) (HX & E, X200), and (**B**) normal histological seminiferous tubule structure with complete spermatogenesis (*). Note: Leydig interstitial cell between seminiferous tubules (arrow) (H & E X 400).

AFP treatment severely damaged the testes, leading to the complete absence of spermatogonia, primary and secondary spermatocytes, spermatids, spermatozoa, and the spermatogenesis process (Figure 3A, B). Moreover, the seminiferous tubules were shrunken, degenerated, and distantly separated (Figure 3A), which could be associated with the buckling of their basement membranes. Atrophy and necrosis of the seminiferous tubules were characterized by the thickening of the basement membrane and Leydig cell degeneration with deeply stained pyknotic nuclei (Figure 3B).

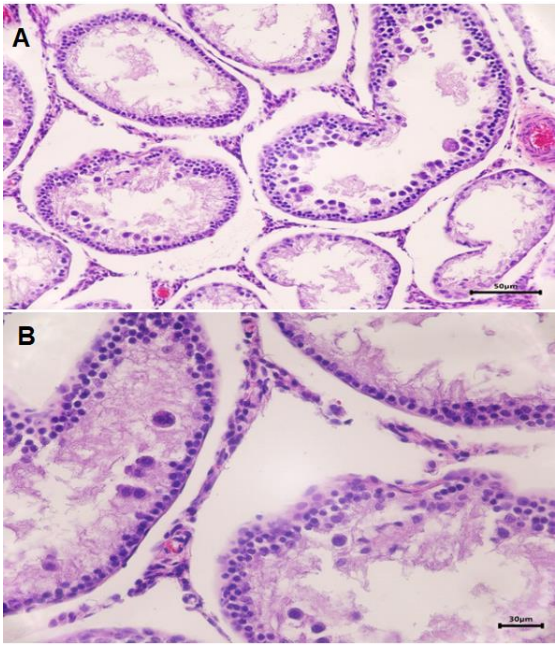


**Figure 3.** Microscopy of AFP-treated rat testis presenting (**A**) shrinkage, degeneration, and necrosis of seminiferous tubules lining spermatogenic cells (arrows) (HX & E X 200), and (**B**) degeneration and reduced numbers of seminiferous tubules lining spermatogenic cells (arrows). Note: Leydig interstitial cell degeneration with deeply stained nuclei (*) (H & E X 400).

The testes of AFS-treated rats were disorganized, with detached spermatogenic and germinal epithelium of the seminiferous tubules. Desquamative germinal cells were observed in the seminiferous tubule lumen (Figure 4A). The extent of the histological changes confirmed complete degeneration of the spermatogenic epithelium of the seminiferous tubules after AFS treatment (Figure 4B).

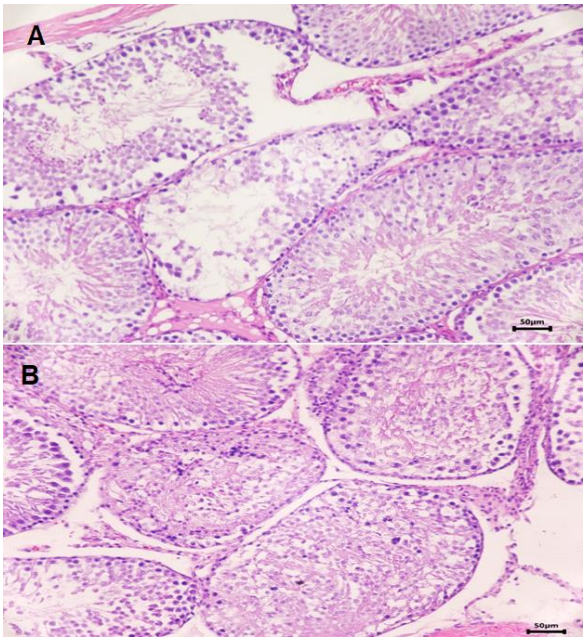


**Figure 4.** Microscopy of AFS-treated rat testis demonstrating (**A**) spermatogenic cell degeneration, reduced numbers (arrows), and destruction of the basement membrane of the seminiferous tubules (*) (HX & E X 200), and (**B**) degeneration of spermatogenic cells (arrows) and Leydig cell degeneration and necrosis (*) (HX & E X 400).

Lung lobules are the structural and functional units of a rat's respiratory tract. Respiratory bronchioles, alveolar ducts, and alveolar sacs are the main parts of each lobule. Thin connective tissue layers separate the lobules. A single cuboidal epithelium layer composed of ciliated and Clara cells lines the bronchioles, and alveolar ducts branch off from the respiratory bronchioles (Figure 5A, B). Alveolar sacs are clusters of alveoli, which are lined with a flat epithelium surrounded by a dense capillary network. The alveolar epithelium contains two types of cells: respiratory type I, or flat cells,

and type II, or large cells (Figure 5A, B). Adjacent alveoli connect through the interalveolar septum, containing macrophages, fibroblasts, mast cells, leukocytes, and lymphocytes (Figure 5B).

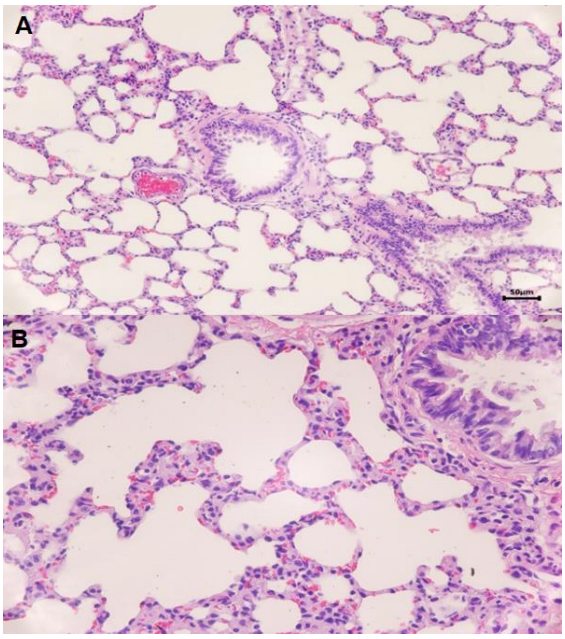


**Figure 5.** Photomicrographs of control rat lung depicting (**A**) normal lung lobule with respiratory bronchioles (RB), alveolar duct (AD), and lung alveoli (A) (HX & E, X 200), and (**B**) normal lung lobule containing respiratory bronchioles with epithelium, alveolar sac (*), and lung alveoli (A). Note: the interalveolar septum contains blood capillaries and connective tissue cells (arrows) (HX & E, X 400).

Treatment of rats with aflatoxin B1 powder significantly changed the lungs in the AFP group. The damage included infiltration of perivascular inflammatory cells, mast cell accumulation, dilated alveolar sacs, and thinning of the alveolar wall (Figure 6A, B). Some specimens presented thickening of the alveolar wall and increased interlobular connective tissue, which is associated with mast cells and oedema (Figure 6C). Mast cells are specialized internal environment tissue cells which actively participate in inflammatory, regulatory, and protective functions through special mediators.

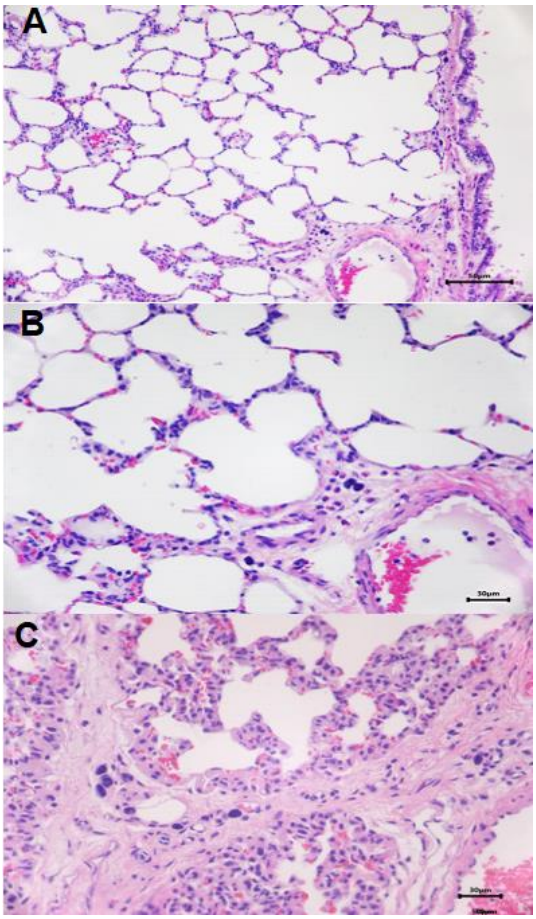


**Figure 6.** Photomicrographs of AFP-treated rat lung revealing (**A**) thinning of the alveolar wall (arrows) (HX & E, X 200), (**B**) thinning of the alveolar wall (arrows), perivascular oedema (E), and mast cell accumulation (thick arrow) around the blood vessels (HX & E, X 400), and (**C**) thinning of the alveolar wall (arrows), oedema (E), increased interlobular connective tissue, and mast cell accumulation (thick arrow) around the blood vessels (HX & E, X 400).

The AFS-treated group presented alveolar degeneration with significant haemorrhaging from the capillaries in the interalveolar septa. Mass destruction of cellular areas involved in alveolar wall synthesis and necrotic cell accumulation were observed as well. In addition, oedema and mast cell accumulation in the interlobular connective tissue and the perivascular area were noted. AFS treatment also resulted in perivascular inflammatory cell infiltration and increased interlobular connective tissue (Figure 7A, B, C, D). Some alveolar walls exhibited thinning associated with

emphysema (Figure 7E), while the pulmonary blood vessel walls were thickened with haemolysed blood (Figure 7E).

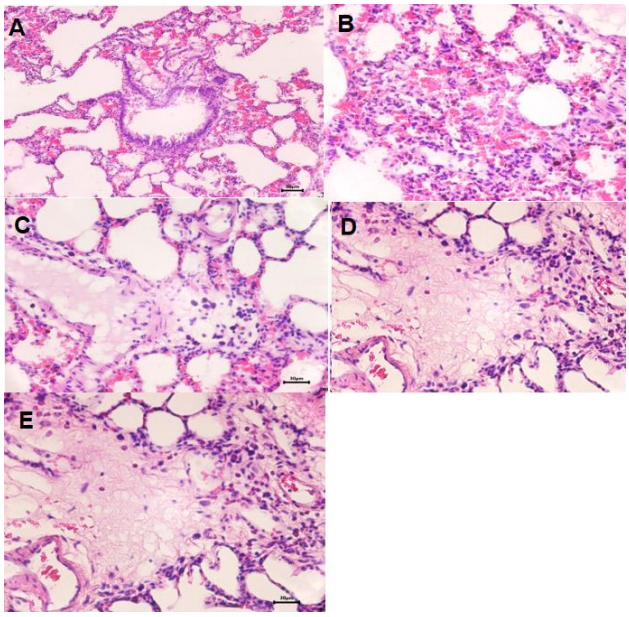


**Figure 7.** Photomicrographs of AFS-treated rat lung exhibiting (**A**) alveolar degeneration and congestion of interalveolar septa with blood (HX & E, X 200), (**B**) alveolar degeneration, inflammatory cell infiltration, and congestion of interalveolar septa with blood (HX & E, X 400), (**C**) alveolar degeneration, inflammatory cell infiltration (*), perivascular oedema (E), and mast cell accumulation (arrow) (HX & E, X 400), (**D**) inflammatory cell infiltration (*) and congestion of the blood vessels (BV) (HX & E, X 200), (**E**) alveolar degeneration, inflammatory cell infiltration (*), and perivascular oedema (E) (HX & E, X 400).

***HPLC of liquid (AFS) and powder (AFP) phases of aflatoxin B1***

Reverse-phase HPLC is commonly used for the detection and separation of aflatoxins. In this study, aflatoxin B1 was subjected to high-precision and high-sensitivity HPLC. Elution was achieved with $H_2O$ (80%) and MeOH (20%) (v/v) at a flow rate of 1 ml/min. Aflatoxin B1 detection was performed at a wavelength of 375 nm. Aflatoxin B1 (100 µl) was injected into the HPLC chromatograph, and AFS was observed at 375 (Figure 8). HPLC also presented a spiked aflatoxin

B1 (AFP)-specific chromatogram at 375 nm (Figure 9). The retention time of both chromatograms was 10 min. HPLC peaks were sharp, with high standard concentration and increased area.

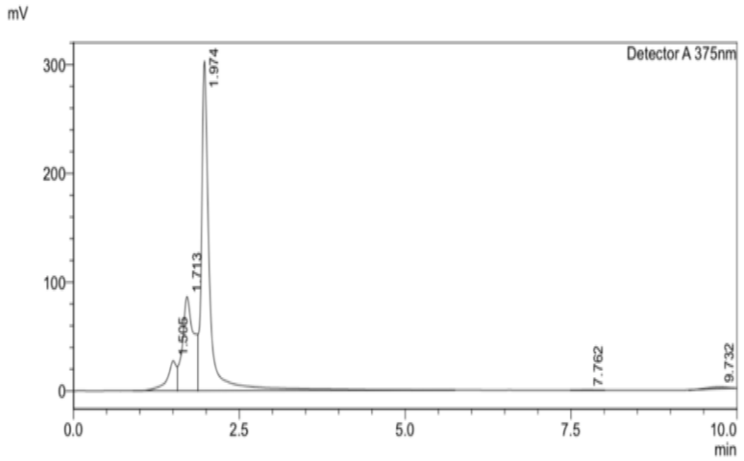


**Figure 8.** HPLC chromatogram of liquid phase aflatoxin B1 (AFS) at 375 nm and respective retention times

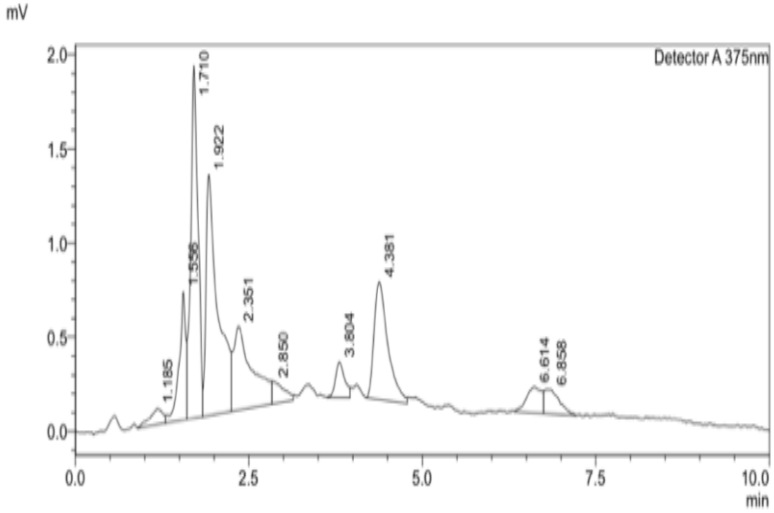


**Figure 9.** HPLC chromatogram of powder phase aflatoxin B1 (AFP) at 375 nm and respective retention times

***GC of liquid (AFS) and powder (AFP) phases of aflatoxin B1***

Gas chromatography is also commonly applied for the detection and separation of aflatoxins. The initial temperature of the injector and column was lower than 60°C to prevent the broadening of peaks, and a longer column facilitated the separations. Rapid heating and a high flow rate of the carrier gas (He) is required for higher sensitivity. Therefore, the initial and final temperatures were set at 50 and 250°C, respectively, and the heating rate was set at 15°C/min with a carrier gas flow rate of 2 ml/in. GC chromatograms of AFS and AFP at 272 nm are presented in Figure 10 and Figure 11, respectively. The retention time of both chromatograms was 9 min. Aflatoxin B1 identities were confirmed by the retention time. Peak purity was confirmed by measuring a qualifier as an internal standard.

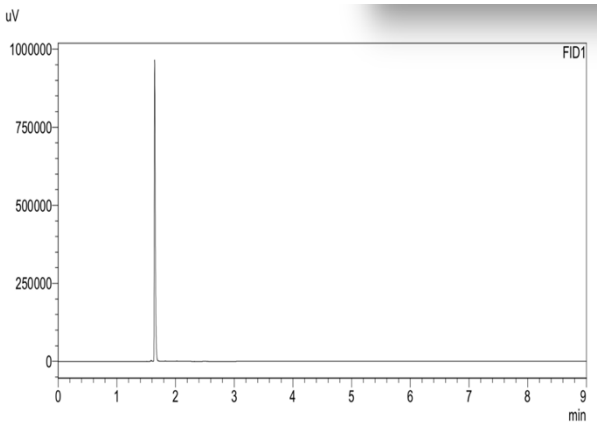


**Figure 10.** GC chromatogram of liquid phase aflatoxin B1 (AFS) at 272 nm. AFB1, Column: DB-5, 0.25 mm i.d.x10 m. Temp. programme: 50–250°C, 15°C/min.

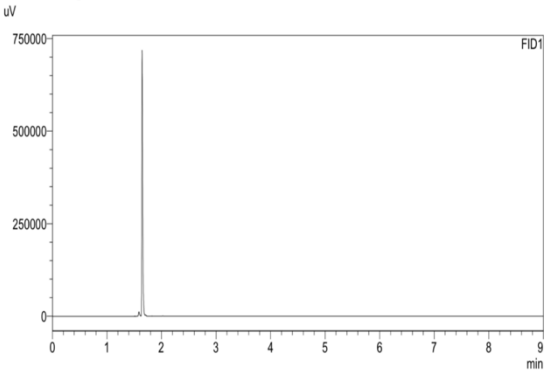


**Figure 11.** GC chromatogram of powder phase aflatoxin B1 (AFP) at 272 nm. AFB1, Column: DB-5, 0.25 mm i.d.x10 m. Temp. programme: 50–250°C, 15°C/min.

**DISCUSSION**

The present study explores the effects of aflatoxin B1 (powder and liquid) on haematological parameters, oxidative stress markers, and lung and testis tissues of adult male albino rats. The results showed insignificant body weight changes (days 1, 7, 14, and 21) between the AFP, AFS, and control groups. However, weight gain and weight gain percentage were significantly higher in the AFS group than in the AFP group. Khaled and Thalij (2021) have reported that feeding rats maize and maize products contaminated with aflatoxin B1 for 21 days significantly reduced their body weight and rate of weight gain. Supriya et al. (2014) reported significantly decreased weight gain in AFB1-exposed rats (10, 20, and 50 mg/kg body weight).

The present study revealed insignificant differences in RBC count, haemoglobin content, MCV, MCH, and platelet counts between the control, AFP, and AFS groups. Khaled and Thalij (2021) reported significantly reduced blood parameters (RBCs and haemoglobin content) in rats fed an aflatoxin B1-treated diet (39.5 μg/kg) as compared to the control group, whereas the differences were insignificant at lower AFB1 concentrations (32.7, 37.9, and 29.0 μg/kg). Similarly, Ramamurthy and Rajakumar (2016) demonstrated reduced RBC counts and haemoglobin content in aflatoxin-fed animals in comparison to controls. The decreased RBC levels in rats could be related to aflatoxin-associated anaemia involving the down-regulation of the activity of erythropoietin hormone, which is mainly secreted by the kidneys and (to a lesser extent) the liver. The low haemoglobin level may be linked to decreased erythrocyte volume, defective haem biosynthesis in the bone marrow, or reduced erythropoietin formation (Khaled and Thalij, 2021).

Neutrophil, lymphocyte, monocyte, and basophil counts were significantly increased in the AFP and AFS groups as compared to controls. A significant rise in the eosinophil count was noted in the AFP group relative to the control, while a significant increase in the lymphocyte count of the AFS group was observed in comparison to AFP-treated rats. Khaled and Thalij (2021) reported significantly increased WBC counts in rats fed aflatoxin B1 at various concentrations (39.5, 37.9, 32.7, and 29.0 μg/kg) as compared to the control group. Ramamurthy and Rajakumar (2016) have also revealed an aflatoxin treatment-associated increase in WBCs as compared to controls. The rise in WBC count, mainly consisting of neutrophils, could be due to their inflammatory response (Khaled and Thalij, 2021).

MDA is commonly used to determine the degree of lipid peroxidation (LPO) (Sezer et al., 2020). ROS-induced removal of a hydrogen atom from unsaturated fatty acid chains yields MDA during the LPO process (Etem et al., 2014). In the present study, aflatoxin administration (AFP and AFS) for 21 days significantly increased MDA and NO serum levels in comparison to the control. This phenomenon could be linked to enhanced ROS levels under the stress of AF intoxication. MDA and NO serum levels were higher in the AFS group than in the AFP group, which indicates more oxidative stress in the AFS group. AFB1 has been reported to promote LPO in mouse testes (Farombi et al., 2005; Verma and Nair, 2002). The release of free radicals (*in vitro* and *in vivo*) may mediate AFB1-based cell injury and initiate toxic lipid peroxidation, which damages the fatty acids of cell membranes (Cao et al., 2022). Increased MDA and NO levels in AFB1-treated rats are associated with relentless free-radical production and incapacitated immunity against antioxidants. Shen et al. (1994) demonstrated a significant dose-dependent rise in MDA levels and conjugating dienes in response to AFB1 treatment. AFB1 has been shown to initially induce LPO-based alterations,

whereas GSH metabolism and antioxidant enzymes mediate the toxicity (Rastogi et al. 2001, Abdel-Daim et al., 2020). Aleissa et al. (2020) reported increased DNA, RNA, and cholesterol and lower glycogen levels in AFB1-treated animals. Oxidative damage refers to impairment of the functioning of cellular components (membranes, nucleic acids, proteins, and enzymes) by reactive oxygen species ($H_2O_2$, hydroxyl free radicals ($OH^-$), and superoxide radicals ($O2^-$)). These agents extract a hydrogen atom from polyunsaturated fatty acids, causing lipid peroxidation to initiate cellular injury (Pu et al., 2021).

In this study, significantly decreased testosterone serum levels were noted in AFP and AFS-treated rats as compared to controls. However, these changes were insignificant between the AFP and AFS groups. These results demonstrated aflatoxin toxicity to the testes. Leydig cells produce testosterone, which is necessary for testicular function and spermatogenesis (Walker, 2011). Decreased testosterone synthesis restricts spermatogenesis in rodents and men, leading to reduced sperm density (Walker, 2011). Multiple studies have reported that decreased serum testosterone levels led to reduced sperm concentration in AFB1-treated mice (Abu El-Saad and Mahmoud, 2009; Mathuria and Verma, 2008; Ibrahim and Salim, 1994; Egbunike et al., 1980). Supriya et al. (2014) exposed rats to various doses of AFB1 (10, 20, and 50 mg/kg body weight), which significantly decreased serum testosterone levels in comparison to control rats. Testosterone production, which is necessary for sperm development in adult mammals, may be disrupted by AFB1 exposure (Adedara et al., 2014; Abdel-Aziem et al., 2011). Verma and Nair (2002) observed a significant rise in cholesterol concentrations in the testes of AFB1-treated mice, possibly due to incomplete cholesterol utilization and impaired steroidogenesis.

Histological examination revealed severe damage and the complete absence of spermatogonia, primary and secondary spermatocytes, spermatozoa, and spermatids in the AFP-treated group. Moreover, AFP toxicity led to atrophy and necrosis of the seminiferous tubules, characterized by Leydig cell degeneration with deeply stained pyknotic nuclei and a thickened basement membrane. The histological examination of the testes of AFS-treated rat revealed detachment of the germinal and spermatogenic epithelium of the seminiferous tubules, followed by its complete degeneration. AFS-treated rats presented more obvious damage than AFP-treated rats. Kudayer et al. (2019) reported that aflatoxin B1 administration to rats for 7 days resulted in excessively vacuolated testicular cells and suppressed spermatogenesis. Other studies have also revealed harmful aflatoxin-related effects on the reproductive system (Murad et al., 2015; Verma and Nair, 2002). Aflatoxin B1 induces pathological alterations in the testes through degeneration and necrosis of the epithelial lining of the seminiferous tubules (Murad et al., 2015). Aflatoxin B1 is also known to pathologically alter sperm mitochondria and the extrusion of outer dense fibres (Faisal et al., 2008). Ingestion of aflatoxin B1 may result in infertility due to increased morphological abnormalities in sperm cells and reduced sperm cell viability and counts (Kudayer et al., 2019). Cao et al. (2017) reported a significant AFB1-associated reduction in the testes index and in sperm quality and concentration. They also noted significant AFB1-based disruption of testicular function and sperm production (Abu El-Saad and Mahmoud, 2009). Oxidative stress and direct toxicity to macromolecules are considered major AFB1 cytotoxicity-related mechanisms (Towner et al., 2002). Highly enriched polyunsaturated fatty acids make spermatozoa and testes susceptible to oxidative stress (Farias et al., 2010). Oxidative stress is

known to reduce sperm quality and is widely accepted to be a mechanism of AFB1 cytotoxicity to the testes (Yu and Huang, 2015; Abdel-Aziem et al., 2011). AFB1 toxicity causes the overproduction of free radicals and suppression of antioxidant enzyme-based defence systems, leading to testicular damage and low sperm concentration and quality (Abdel-Aziem et al., 2011; Abu El-Saad and Mahmoud, 2009).

Histological lung examination revealed obvious changes in AFP-treated rats. The damage appeared mainly as infiltration of perivascular inflammatory cells, mast cell accumulation, alveolar sac dilatation, and alveolar wall thinning. An increase in oedema-associated interlobular connective tissue was observed as well. The lungs of the AFS group presented alveolar degeneration with marked capillary bleeding, alveolar cell wall destruction, and necrotic cell accumulation. In addition, mast cell accumulation and oedema were noted in the interlobular connective tissue and perivascular area. Infiltrations of perivascular inflammatory cells were observed as well. Some alveolar walls exhibited thinning of the alveolar wall, which is associated with emphysema, while the walls of some pulmonary blood vessels were thickened with haemolysed blood. These findings are in agreement with previous studies reporting aflatoxin toxicity to the respiratory systems of humans and rats (Massey et al., 2000; Larsson and Tjälve, 2000; Lee et al., 2016). Kudayer et al. (2019) found that oral AFB administration (0.5 mg/kg for 7 days) resulted in inflammatory cell infiltration to lung tissues followed by exudates containing dilated alveolar sacs. Food industry employees are often exposed to aflatoxin B1 by inhaling aflatoxin-contaminated dust during the shelling and processing of grains (Larsson and Tjälve, 2000). The nasal mucosa can convert aflatoxin into active metabolites (Larsson and Tjälve, 2000). Aflatoxin B1 also induces pathological changes such as lung cancers and pulmonary adenomas (Coulombe Jr, 1994). Lung susceptibility could be due to the low aflatoxin B1 degradation capacity of glutathione S-transferase in the lungs (Stewart et al., 1996).

Toxic and carcinogenic aflatoxins are secondary metabolites of two different *Aspergillus* species (*A. parasiticus* and *A. flavus*) (Wacoo et al., 2014). The present study undertook HPLC and GC quantitation of aflatoxin B1 (AFS and AFP) in liquid mycelium culture media. Both techniques confirmed aflatoxin production by producing relevant spectra of aflatoxin B1 (AFS and AFP). The study revealed that AFS was more toxic than AFP. The retention time confirmed the detection of aflatoxin B1. Several studies have followed chromatographic methods to detect aflatoxin concentrations in milk, baby food, dairy products, spices, coffee beans, wheat, dried figs, maize, cereals, and peanuts (Shabeer et al., 2022). A highly accurate and sensitive HPLC method can reliably quantify aflatoxins in food commodities (Miklós et al., 2020). The combination of HPLC and mass spectroscopy is known to accurately assess aflatoxin concentrations in milk (Maggira et al., 2021). Escrivá et al. (2017) used HPLC and LCMS (liquid chromatography-mass spectrometry) to estimate aflatoxin concentrations in baby food within a range of 0.003–0.008 μg/L. The carcinogenic and mutagenic potential of aflatoxin $B_1$ (AFS) has also been established through GC and HPLC methods.

It has been suggested that passive diffusion is a possible mechanism of aflatoxin absorption by mucous membranes (Gallo et al., 2008), in which the physical state of the toxin plays a role in the concentration absorbed by cells, which may reflect the severity of the damage. This was observed in the current study, which showed significant differences between the damage caused by the powder aflatoxin (AFP) and the liquid aflatoxin (AFS), with AFS inducing more severe damage. The role of

the physical state of the toxin on the concentration absorbed by the mucous membrane requires further investigation.

## CONCLUSION

This study compared the effects of administration of aflatoxin powder (AFP) and aflatoxin liquid (AFS) on the testes and lungs of rats and explored their potential mechanisms of action. It appears that the physical state of AFB1 (liquid/powder) makes no difference in terms of body weight change, and no changes in RBCs were observed in animals from the two treatments. On the other hand, a significant increase in WBC count was observed, which may be due to the inflammatory response due to aflatoxin effects. Elevated oxidative stress markers and reduced serum testosterone levels in response to the AFP and AFS treatments were recorded, with significant differences between AFP and AFS. Both AFP and AFS severely damaged the testes and lung tissues, with AFS toxicity shown to be significantly higher than that of AFP. The association between the physical state of the toxin (powder/liquid) and the concentration absorbed remains to be fully investigated. Policymakers should devote greater attention to preventing contamination of the food chain with aflatoxin B1.

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