

# Assessment of Rats Exposed to Aflatoxin B1 Early in Life and Impact on the Offspring

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**Abstract:** Aflatoxin-producing *Aspergillus* fungi produce hazardous compounds that often contaminate food in tropical regions. In some areas, the most harmful kind, aflatoxin B1 (AFB1), is a primary contributor to liver cancer, often known as hepatocellular carcinoma, or HCC. There is a significant frequency of aflatoxin contamination, and early onset of HCC has been linked to perinatal exposure to AFB1. The possible consequences of perinatal exposure to AFB1 and possible epigenetic changes which might affect the risk of cancer in the future are investigated in this study.

Two dosages of AFB1 (high: 5 mg/kg, low: 0.5 mg/kg) were administered to pregnant rats before conception, during pregnancy, and during the weaning process to investigate this. Next, these groups were contrasted with a control group that had not been exposed. The babies were kept under observation until they were three weeks or three months old, at which time samples of their liver and blood were taken for examination. Hormones related to reproduction, gonadotropin, and thyroid were assessed after three months, whereas body weights and lipid profiles were determined at three weeks and three months.

The children of high-dose prenatal AFB1 exposure had notable weight reductions: 16.3% at birth, 31.6% at three weeks, and 7.5% at three months, according to the results. The lipid and hormone profiles were likewise changed by both exposure levels. TP53 gene and H19's DNA methylation levels in blood and liver samples were assessed using pyrosequencing. TP53 methylation was markedly elevated in blood samples from rats subjected to both low- and high-doses, but dramatically decreased in liver samples as compared to controls. In contrast, Rats exposed to the high-dose group had increased liver and decreased H19 methylation in their 3-month blood samples.

These findings thus point toward an increased risk of early-onset HCC, whether perinatal AFB1 exposure can lead to overt alterations in hormone, lipid, and weight profiles with modulation of epigenetic regulation. Further studies are needed to completely understand the long-term implications of these modifications leading to cancer development.

**Keywords:** Gestational exposure, AFB1, hormones, lipid, liver, DNA methylation, developmental causes.

## I. BACKGROUND

According to the DOHaD theory, which focuses on the developmental origins of health and disease, our health outcomes might be influenced by the circumstances we encounter during pregnancy and life early stages. According to early research that supported this theory, individuals who, throughout the first two years of their existence, were tiny at birth were far more likely to suffer from ischemic heart disease or coronary heart disease. Quick childhood weight gain and catch-up growth seem to be linked to a higher risk. Over the years, studies have shown that a range of factors, such as the availability of nutrients and chemical exposure, can lead to the development of adaptive responses in the fetus and infant that affect their growth, development, metabolism, and eventually put them at risk for adult disorders. There is now proof that early life circumstances

can raise the risk of cancer, especially hormone-dependent malignancies, in addition to metabolic and cardiac illnesses. Birth weight is frequently used as a predictor of future health and as a stand-in for the effects of the prenatal environment. According to recent research, low birth weight is connected to adult HCC, liver disorders, and hepatoblastoma, while high birth weight is linked to higher mortality from both prostate cancer and other causes. (Gluckman et al., 2008)

Epigenetic modification, which involves chromatin and DNA modifications that control the Regulation of gene activity through mechanisms that do not involve changes to the DNA sequence is a crucial biological mechanism in DOHaD. Epigenetic modifications can be inherited by subsequent generations and, in certain situations, through cell division. A putative mechanism for non-genotoxic carcinogenesis, abnormal

epigenetic regulation involves genes such as Igf2, H19, and Igf1 and is crucial for growth and development during fetal and childhood stages. After fertilisation and implantation, Waves of epigenetic reprogramming make the epigenome particularly susceptible to damage during development. Any disturbances that occur during this reprogramming stage can impact all following cells in various tissues. Early environmental exposures during pregnancy can disturb epigenetic programming, which can lead to the development of disease later in life. One type of steady epigenetic regulation that affects many CpG dinucleotides is DNA methylation. Studies have demonstrated that exposure to chemicals like as phthalates and bisphenols can modify the epigenome of kids. Additionally, mycotoxins that are found naturally, including aflatoxin B1 (AFB1), have been linked to epigenetic modifications. (Sharma et al., 2020)

Molds belonging to the *Aspergillus* species produce aflatoxins as secondary metabolites. Particularly in tropical areas, contaminated foods such grains, nuts, eggs, spices, milk, and meat expose humans to these toxins. Aflatoxin contamination is a worldwide problem that affects portions of Europe and the Americas, despite being most common in these regions. Aflatoxin B1 (AFB1) is the most prevalent and toxic aflatoxin affecting humans. Its metabolite, AFB1-exo-8,9-epoxide, binds to DNA, forming adducts that contribute to liver toxicity and cancer development. Combined exposure to AFB1 and hepatitis B virus significantly increases the risk of liver cancer, the fourth most common cancer in sub-Saharan Africa. In addition to creating DNA adducts, AFB1 has been demonstrated to alter the liver's epigenetic makeup. In Africa, AFB1 is introduced to children during breastfeeding, weaning, and pregnancy. Although one epidemiological study connected early-life exposure to AFB1 to altered DNA methylation and stunted growth, this field of inquiry is still in its infancy and lacks long-term investigations in chronic exposure models in humans or animals. (Zimmermann et al., 2016)

Early carcinogenesis is influenced by epigenetic changes of tumour-inhibiting genes. The well-known suppressor of tumours Tp53, which encodes the p53 protein, is also implicated in liver metabolism and homeostasis, hormone and lipid metabolic pathways, and possibly cancer. AFB1 causes disturbances in the metabolism of fat and hormones in rats, according to earlier research. Cancer risk is also influenced by imprinted genes, including H19 and Igf2, which are well-known for their roles in early development. (Amarger et al., 2017)

Through the use of a rat model, this study seeks to determine how perinatal AFB1 exposure affects hormone levels,

lipid levels, weight, and epigenetic programming.

## II. METHODOLOGY

### Animals

The Covenant University Health Research Ethics Committee approved the study ethically. For the experiment, nine male and total of (eighteen 6-week-old inbred female Wistar rats weighing 100–120 grams were used in the experiment. The animals in the animal home were housed in clean, air-conditioned cages that maintained a regular 12-hour cycle of light and dark and were room temperature. Before the trial started, they were given a typical rat diet, clean water on demand, and three weeks to get used to their surroundings.

### Chemicals

The source of aflatoxin B1 (AFB1) was Sigma-Aldrich, located in St. Louis, Michigan, USA. Biobase (Jinan, China) and Randox (Crumlin, UK) provided the hormone and lipid profile kits, respectively.

### Experimental Design

Three groups, each with six animals, were randomly assigned to contain female rats. Feed containing 0.5 and 5.0 mg/kg of AFB1 was given to them; the third group was used as a control. These dosages were chosen by the levels of AFB1 in maize grains. One male rat and two females were housed together in a cage to enhance mating, and the mating time stopped when a vaginal plug was found. AFB1 therapy was administered as used in other environmental epigenetic research studies. Perinatal exposure is defined as beginning two weeks before mating, continuing during the pregnancy, and concluding three weeks after the progeny are delivered. Throughout the trial, monthly body weight measurements were made to ensure the animals maintained a healthy weight. As used in other environmental epigenetic research studies, perinatal exposure is defined as beginning two weeks before mating, continuing during the pregnancy, and concluding three weeks after the progeny are delivered. Using lithium heparin-coated syringes, blood was drawn via heart puncture, and the resulting plasma was centrifuged for 15 minutes at 3000 rpm to analyse the lipid and hormonal profiles. The entire liver was removed, cleaned in phosphate buffer saline, and stored in the freezer for additional examination. The remaining babies were likewise put to death so that their blood and liver could be collected, after they had been fed a diet devoid of AFB1 for three months.

## Hormones and Lipids Analysis

The following hormone profiles were determined in the plasma using Biobase ELISA kits, as directed by the manufacturer: prolactin, triiodothyronine (T3), thyroxine (T4), follicle-stimulating hormone (FSH), testosterone, progesterone, estradiol, and luteinizing hormone (LH), and prolactin. Microplate wells pre-coated with certain antibodies were filled with samples or standards, followed by the addition of the horseradish peroxidase conjugate and an hour of incubation at 37 °C. Following the incubation period, chromogen was applied to the wells five times to create colour. The absorbance at 450 nm was measured using a plate reader. Randox commercial kits were used to assess total plasma cholesterol and triglycerides.

## DNA Methylation Analysis

Qiagen (Germantown, MD, USA) DNA DNeasy® Blood and Tissue extraction kits were utilised. Following the manufacturer's instructions, high-molecular-weight DNA was extracted from liver and blood samples at three weeks and three months, respectively. DNA samples were transported to Michigan University on dry ice and kept at 80°C until examination. Zymo Research (Irvine, CA, USA) kits were used to treat samples with sodium bisulfite. DNA methylation at Tp53 (9 CpG sites) and H19 (6 sites) at phosphate -cytosine -guanine dinucleotides (CpG sites) was investigated using pyrosequencing. PyroMark Assay Design Software 2.0 (Qiagen) was used to modify primer designs based on previous experiments in order to amplify the areas of the H19 and Tp53 promoters

The H19 assay targets the gene's second imprinting control region (ICR2). For each gene analysed, approximately 50 ng of bisulfite-treated DNA was amplified using Qiagen's HotStarTaq Master Mix. Each experimental batch included four replicates of commercially obtained rat DNA along with appropriate negative controls. Methylation percentages were quantified using the Qiagen PyroMark ID Pyrosequencer. Pyro Q-CpG Software computed the percentage of performed quality control checks and methylation. Only CpG sites and samples that passed the quality check were used in the statistical analysis. The average of the duplicate reads was determined for more than 10% of the samples that were tested twice.

## Statistical Analysis

For every continuous variable (weights, site-specific DNA methylation), descriptive statistics were computed; for every

categorical variable, frequencies were computed. The weight at sacrifice and birth weight of all offspring and sex-stratified offspring were compared for the following comparisons using two-sided t-tests with unequal variances: low vs. high, control vs. low exposure group, and low vs. high. Weight was used as a repeated measure (at birth, three weeks, and three months), and mixed-effects regression was used to account for sex. If testing an interaction term between exposure group and stage improved model fit, a random intercept for the dam was added. The analysis of the hormone and cholesterol data was done with IBM Corp.'s SPSS v23 (Armonk, NY, USA). To compare the means in each exposure group, one-way analysis of variance (ANOVA) with post-hoc tests was employed.

CpG sites and/or samples that failed quality control were eliminated from DNA methylation data. The liver samples collected three months ago had poor DNA quality; hence, they were not included in the analysis. Because Tp53 had stronger intra-site correlations and greater passing rates, data from the first six CpG sites were selected for additional analysis. For statistical analysis, the six CpG sites of H19 were also averaged together and showed a strong correlation. Using ANOVA, the average values for every gene were compared in 3-week livers, 3-week blood, and 3-month blood samples across all exposure groups. If Levene's test revealed that group variances were not homogeneous, Welch's F-test was employed. To capture intra-gene variability at each CpG site, mixed-effects regression was used, with the assumption that Tp53 had an autoregressive covariance structure and H19 had compound symmetry. With the exposure group acting as the predictor, separate models were conducted for the liver data (adjusting for CpG site and sex) and the blood data (controlling for CpG site, sampling time point, and sex). Since litter contributed to the explanation of some of the covariance for H19, a random intercept for litter was incorporated into H19 methylation models. SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses, and p-values less than 0.05 were regarded as statistically significant.

## III. RESULTS

### Rat Population's Weight Status and Weight Gain

The offspring from six dams (unexposed control group), five dams (low dosage exposure group) and four dams (high dose exposure group) were allowed to survive up to three weeks or three months. Weights were obtained at birth and at sacrifice.

Compared with the newborn weight of the control group, that for the high exposure group was 0.8g and 1.3g lower at delivery, respectively, compared with the latter group. At 3 weeks of age, the weights of children of both the low and the high exposure groups were statistically significantly lower irrespective of gender compared with the control group. After three months, the control group's weights remained the highest. Statistically significant differences were noted when the low group was compared with the high group or the low group with the control group. Data on weight from 125 children were modeled as a repeated measure, with exposure group remaining statistically significant upon accounting for sex and stage,  $p < 0.0001$  for the exposure fixed effect and for the interaction of the exposure group by timing.

**Table 1: Offspring Weight (g) and Prenatal Exposure to aflatoxin B1**

Exposure Group	Offspring	At Birth (Mean ± SD)	N	3 Weeks (Mean ± SD)	N	3 Months (Mean ± SD)	N
Control	All	6.3 ± 2.5	41	47.8 ± 5.1	19	191 ± 27.9	22
	Females	6.2 ± 1.5	22	46.1 ± 3.6	11	167 ± 13.7	11
	Males	6.5 ± 1.6	19	50.0 ± 6.1	8	217 ± 12.7	11
0.5 mg/kg	All	6.3 ± 1.2	48	34.6 ± 6.8 **	19	155 ± 12.9 **	29
	Females	6.4 ± 1.1	20	34.9 ± 7.5 *	9	158 ± 11.8	11
	Males	6.3 ± 1.1	28	34.3 ± 6.6 **	10	153 ± 13.3 **	18
5.0 mg/kg	All	5.3 ± 0.6 ** ##	36	32.7 ± 4.3 **	17	177 ± 33.7 #	19
	Females	5.4 ± 0.7 * #	21	33.8 ± 4.6 **	10	153 ± 15.9 *	11
	Males	5.2 ± 0.6 * ##	15	31.1 ± 3.5 **	7	209 ± 23.3 ##	8

$p < 0.05$ , \*\*  $p < 0.001$  in comparison to the control group (all females or all men) throughout the same time period.

### Lipids

In the three-week and three-month-old animal plasma samples, total cholesterol and triglycerides were measured. Triglycerides and cholesterol decreased with age in both exposure groups. At 3 weeks, compared to controls, there was a 32% and 36% reduction in high and low exposure groups for cholesterol levels, respectively ( $p < 0.05$ ) (Table 2). At three months, both exposed groups had 19% less cholesterol than controls,  $p < 0.05$ . At three weeks, when compared to the low dose exposed group, 14%, the high dose exposed group had 18% more triglycerides and this difference was significant,  $p < 0.05$ . At three months, the low-exposure group no longer had significantly higher levels compared with the control group; however, the high-exposure group remained 19% higher in triglycerides compared to the control group. Among rats of both sexes in the high exposure group, there were significantly high triglycerides, and when the male rats in the low exposure group were subcategorised in relation to sex, their levels for triglycerides remained significantly lower, compared to those of the control group, in both time periods.

**Table 2: Liver Concentrations of Triglycerides and Cholesterol by Exposure Group at 3 Weeks and 3 Months of Age**

AFB1 Dose Group	Cholesterol (mg/dL)	Triglycerides (mg/dL)
	3 Weeks	3 Months
Control (no AFB1)	299.26 ± 19.83 a	184.59 ± 11.98 a#
0.5 mg/kg	204.89 ± 17.46 b	149.01 ± 9.82 b#
5.0 mg/kg	190.59 ± 9.99 b	151.23 ± 9.56 b#
The figures are Mean ± SD. At $p < 0.05$ , values in the same column with different superscripts (a, b) differ significantly. # denotes a significant difference, at $p < 0.05$ , from three weeks earlier within the same exposure group.		

### Three Months of Age: Hormonal Changes

We determined that there were significant differences in thyroid, gonadotropic, and reproductive hormone levels among three-month plasma samples, which were categorised by sex. Testosterone levels in men randomised to low  $p 0.1$  and high  $p 0.05$  were

lower than their control group, and low exposure males had higher estradiol and lower prolactin than the control and high exposure groups. Females in both groups had lower levels of LH p 0.05 than controls. Progesterone levels were only lower in females in the high exposure group, while those in the low exposure and high exposure groups were lower in prolactin and progesterone. Female high exposure group p t 0.04 vs low dose and control group. T3 drop of 0.01, low exposure group of males 0.01.

### Methylation of DNA in Blood and Liver

Tables 4 and 5 exposure-dependent methylation varies over time, see tables 4 and 5. Liver methylation of TP53 and h19 DNA at 3 weeks of age is shown in Table 3. Both groups showed a decrease in TP53 methylation in the liver compared to the unexposed group, with anova p-value of 0.06. Remember the 9 CPG sites of TP53. Remember, methylation of tp53 was significantly lower in both exposure groups, with estimates of sex and cpg sites being 0.91, 0.34, p 0.01 for high. and 0.09 0.34, p 0.002 for low H19 methylation was higher in liver samples from the low and high exposure groups than in the controls, with a mean value of 0.0041. 5.37 2.56, probability of a positive outcome of acupuncture, p 0.04, and high probability of acupuncture, all adjusted for dam, sex, and site, showed that the differences between the exposure groups remained when repeated measures linear regression was applied to six cpg sites.

Table 3: CpG site DNA methylation (%) in rat liver at three weeks of age

Gene	CpG Site	Control	0.5 mg/kg	5.0 mg/kg	ANOVA p-value
Tp53	1	6.7 ± 2.3	4.8 ± 1.6	5.2 ± 1.8	0.066
	2	12.1 ± 4.5	9.6 ± 4.7	10.2 ± 4.9	0.28
	3	17.3 ± 3.8	13.4 ± 4.3	14.7 ± 3.1	0.014
	4	9.7 ± 2.7	6.4 ± 2.5	6.6 ± 1.8	0.0011
H19	1	4.5 ± 1.5	8.3 ± 3.5	8.6 ± 2.6	0.001
	2	3.2 ± 1.1	7.1 ± 2.7	6.5 ± 3.3	0.0034
	3	2.9 ± 0.9	6.9 ± 2.2	7.3 ± 3.5	0.0009
	4	3.7 ± 1.2	8.8 ± 2.7	8.4 ± 2.6	<0.0001

### Targeted mRNA Expression

Livers of 3-week-old children revealed mutations in crucial genes associated with lipid metabolism, inflammation, and cell cycle control Table 4. Offspring exposed to 0.5 mg/kg showed higher levels of H19 and Igf2 mRNA than the control group, while offspring subjected to 5.0 mg/kg showed lower Tp53 expression. At three months of age, there were still alterations in gene expression, most notably a decrease in Tp53 expression.

Table 4: Relative mRNA Expression in Liver by Exposure Group

Gene	Control (mean ± SD)	0.5 mg/kg (mean ± SD)	5.0 mg/kg (mean ± SD)
H19	1.00 ± 0.16	1.58 ± 0.28 *	1.72 ± 0.33 **
Igf2	1.00 ± 0.14	1.29 ± 0.26 *	1.24 ± 0.29
Tp53	1.00 ± 0.12	0.88 ± 0.09	0.73 ± 0.10 **

\*\* p < 0.01 and p < 0.05 about the control group.

## IV. DISCUSSION

We investigated the impact of two aflatoxin b1 afb1 doses administered to rats during pregnancy. We found that the offspring had major effects three weeks after weaning and three months after that. Even after exposure had ended, these effects remained. Reduced weight at birth, three weeks, and three

months, changed lipid profiles (lower cholesterol at three weeks and three months, higher triglycerides at three weeks), and changed hormone levels (lower testosterone, progesterone, and LH) were all linked to perinatal AFB1 exposure. (Benkerroum, 2020) Liver samples collected three weeks apart from afb1 exposure showed higher methylation levels at the h19 ICR and

lower methylation levels at the TP53 promoter. We found that there was a decrease in H19 methylation at three months and an increase in Tp53 methylation at three weeks and three months in blood samples. (Xue et al., 2020)

According to our research, prenatal exposure to AFB1 can lower birth weight and have an impact on weight gain for up to three months of life. Previous studies have shown that pregnant rats exposed to AFB1 had a dose-dependent decrease in their birth weight, and these findings align with them. Analogous patterns have been noted in human investigations, wherein the weight and height of infants during their first year of life were negatively correlated with the mother's AFB1 levels. Additionally, some research has suggested that low birth weight may raise an adult's chance of developing liver disorders and hepatocellular carcinoma (HCC). A major cohort study in daemon found a strong correlation between low birth weight and primary liver cancer in men, and a Finnish birth cohort study found a strong correlation between small birth size and adult non-alcoholic fatty liver disease. (Nazhand et al., 2020)

Afb1 prenatal exposure to triglycerides and cholesterol in rats after weaning and until they reach adulthood has been examined for the first time. At three weeks and three months, we observed that exposure to AFB1 raised triglyceride levels; however, there was no correlation between higher triglyceride levels and body weight. The conditions metabolic syndrome, insulin resistance, and coronary heart disease may be affected by this hypertriglyceridemia. Afb1 exposure results in higher cholesterol at 3 weeks and 3 months, compared to afb1 exposure. This shows that exposures to AFB1 throughout pregnancy and adulthood may have different effects on its cytotoxic mechanism. (Gruber-Dorninger et al., 2019)

The manufacturing of steroid hormones depends on cholesterol. According to our research, prenatal exposure to AFB1 dramatically lowered progesterone and testosterone levels, most likely as a result of lower cholesterol. Sex-dependent steroid hormone reduction may have resulted from AFB1's impact on developmental stage- and sex-specific hormone production. Previous studies have shown that AFB1 exposure during pregnancy can decrease testosterone levels in male rats by affecting androgen biosynthesis-related enzymes. Since LH and FSH control the production of steroid hormones, lower progesterone levels in female rats may be related to lower LH levels. (Zhang et al., 2016)

In female rats, our investigation revealed a drop in LH levels, but no difference in FSH levels between the sexes. Due to

potential changes in exposure timing, these results vary from another study where prenatal AFB1 exposure raised LH and FSH levels. It has also been demonstrated that AFB1 reduces LH in nursing buffalo and adult rats. Furthermore, prolactin and thyroxine levels were reduced in utero exposed to AFB1. Because of its changed hormonal state, AFB1 may be an endocrine disruptor and raise the chance of developing non-communicable diseases in later life. (Deng et al., 2018)

AFB1 has been identified as an epigenetic modulator in addition to a genotoxic agent. Cancer is characterised by epigenetic modifications, such as globally, hypomethylation and hypermethylation of tumour suppressor genes. In vitro models or clinical tumour samples have been used in the majority of investigations on AFB1 and the epigenome. Pregnancy-related exposures on epigenetic programming and cancer risk in adulthood have been demonstrated. Our study is the first to look into whether early exposure to AFB1 changes the epigenome in later life and raises the risk of cancer development, including HCC. Both the liver and the blood samples showed altered DNA methylation at the Tp53 and H19 genes, with varying methylation patterns in these tissues. (Benkerroum, 2020)

Overall, exposure to perinatal AFB1 may impact long-term health outcomes by having a lasting impact on hormone levels, lipid profiles, weight, and DNA methylation.

## V. CONCLUSIONS

A study looked at the impact of daily exposure to AFB1 from conception to nursing and found that the offspring at 3 weeks and 3 months were significantly impacted. Afb1 reduced body weight and altered lipid and hormone levels, and lasted even after prenatal exposure was terminated. These disturbances may raise the chance of developing diseases in later life, which emphasises the need for more investigation into the underlying mechanisms.

It was discovered that the liver and blood samples of rodents exposed to radiation had different levels of DNA methylation at H19 and Tp53, two critical genes for cancer risk. Notably, the direction of these methylation changes was opposite in the liver compared to the blood, underscoring the need for caution when interpreting studies that rely solely on blood samples.

Future research should include epigenome-wide analyses combined with transcriptomics, and long-term monitoring of rodents is also essential and human populations to identify genes

reprogrammed by AFB1 exposure that may contribute to liver cancer and other cancers. This knowledge is essential for informing policy and risk assessments regarding AFB1 contamination in food, particularly for vulnerable populations such as pregnant women and children.

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