TRANSGENERATIONAL EFFECTS OF AFLATOXIN B_1 ON THE NEMATODE *CAENORHABDITIS ELEGANS*

by

KABIRU SUNMOLA

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Understanding aflatoxin B_1 (AFB_1) toxicity has been of major interest since it's a common contaminant in agricultural food crops and also an acute genotoxic and carcinogenic agent in humans. Though previous studies extensively described AFB_1 toxicity in many model systems, no study shows transgenerational effects of AFB_1 *in vivo*. We explored the effects of AFB_1 treatment on growth, generation time, and brood size in F_0, F_1 and F_2 generations of *Caenorhabditis elegans*. AFB_1 exposure exhibited a dose-dependent reduction in brood size, growth, and mean generation time in the F_0 generation. A dose-dependent reduction in body length and brood size was observed in F_1 *C. elegans* and the mean generation time extended significantly ($p < 0.01$) in the highest dose group. F_2 generation results showed less effects on growth and reproduction. Data from this thesis will provide new insight of adverse health effects of potential AFB_1 exposure in animals and humans.

INDEX WORDS: Alternatives to animal testing, Transgenerational effects, Reproductive and Developmental Toxicity, Aflatoxin B_1, *Caenorhabditis elegans*

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DEDICATION

This thesis is dedicated to my dad, Idowu Sunmola, the greatest man I know, to my benefactor and mom, Funke Tijani, and to my siblings for their unconditional support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Aflatoxins

Aflatoxins (AFs) initially gained prominence as a public health issue in the early 1960s after a mysterious "Turkey X" disease killed as many as 100,000 turkeys in England (Blount, 1961; Forgacs & Carll, 1962). Turkey X disease was linked to a peanut (groundnut) meal consumed by the birds contaminated with *Aspergillus flavus* and this led to the naming of the toxic contaminants as aflatoxins (*A. flavus* toxins) (Bennett, Kale, & Yu, 2007). The results of post-morterm examination of animals affected in the Turkey X disease episode showed liver hemorrhages, liver necrotic lesions, and frequently swollen (engorged) kidneys. Histopathological examination also revealed degeneration of the liver parenchyma cells and extensive proliferation of the bile duct epithelial cells (Goldblatt, 2012). Studies in humans also revealed similar outcomes in the liver as a result of ingestion of aflatoxin-contaminated maize (Shank, 1981). AFs are secondary metabolites or mycotoxins produced by toxigenic fungi *A. flavus* and *A. parasiticus* (Roze, Hong, & Linz, 2013). AFs are mainly produced by these fungi growing on food crops like maize, peanuts, rice and cotton seed (Bandyopadhyay, Kumar, & Leslie, 2007) on the field before harvest or under unfavorable storage conditions after harvest. Conditions that favor the production of AFs vary from high insect activity and high temperature on the field to warm temperature and high humidity during storage. Specifically, temperatures that favor the production of AFs fall between 24 and 35°C while moisture content exceeding 7% causes their formation within many food commodities (Williams *et al*., 2004). Some other

conditions that favor the production of AFs on the field and during postharvest include rodent activity as well as inadequate drying of crops (Shuaib *et al*., 2010). Other food commodities associated with AF production as a result of growth of the two previously mentioned species of *Aspergillus* on them include sorghum, chili, soybean, cassava, yam, wheat, melon seeds.

Food products from animals including eggs, cheese, milk and milk products were also prove to be source of AF contamination (Richard *et al*., 2003; Williams *et al*., 2004). This occurs when feed stock of animals like poultry and cows get contaminated due to AF production by fungi present on them. The four main AFs that are naturally found in foods are AFB_1, AFB_2, AFG_1 and AFG_2 with the letters B and G representing colors blue and green, respectively. These are the colors in which toxin isolated from *A. flavus* appeared when exposed to ultraviolet light and the numbers 1 and 2 represent decreasing relative fluorescence values on silica gel thin-layer chromatography (Bennett *et al*., 2007). Other important AF types are aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) which are the hydroxylated derivatives of AFB_1 and AFB_2, respectively, after the two latter types of AFs have been partially metabolized in some animal species like dairy cattle. AFM_1 and AFM_2 have been detected in milk of animals previously exposed to AFB_1 and AFB_2 in their food (Dhanasekaran *et al*., 2011).

Aflatoxin B_1

AFB_1 (Figure 1) is the most important and the most common naturally-occurring carcinogen (Roze *et al*., 2013). It is a very potent genotoxic agent as well (Kew, 2013). AFB_1 has also been identified to be both a hepatotoxic (Chen *et al*., 2010; Groopman, Wang, & Scholl, 1996) and mutagenic (Wang & Groopman, 1999; Mace *et al*., 1997) agent. According to the classification by the International Agency for Research on Cancer (IARC), AFB_1 is a class 1 human carcinogen (Humans, 1993; Wang & Groopman, 1999) meaning it is carcinogenic in

humans. Humans and animals get exposed to AFs via two major routes: (1) by direct ingestion of AF-contaminated foods or by ingestion of dairy products like milk and cheese as well as animal tissues containing AFM_1, the toxic metabolite of AFB_1 (Agag, 2004), or (2) by inhaling dust particles that contain AFs, especially AFB_1, in contaminated foods in industries and production facilities (Coulombe Jr, Eaton, & Groopman, 1993). In the body, AFs are absorbed across the cell membranes and get into blood circulation. As a result, they are distributed to different tissues of the body and to the liver which is the main organ of metabolism of xenobiotics (Bbosa *et al.*, 2013). In the liver of humans and susceptible animals, AFB_1 can be metabolized by cytochrome P450 microsomal enzymes through epoxidation into a reactive epoxide intermediate, AFB_1-8,9-epoxide that binds to DNA which causes DNA damage, and also binds to serum albumin in blood forming protein adducts or can get hydroxylated to become AFM_1 (Wild & Montesano, 2009). DNA damage by AFB_1 is very important because it leads to formation of DNA adducts and gene mutations which is what makes the mycotoxin a potent carcinogenic agent. Some other microsomal enzymes also metabolize AFB_1 through hydroxylation, demethylation and hydration to form AFQ_1, AFP_1, and AFB_{2a} respectively. AFM_1 and AFQ_1 are both formed from the hydroxylation of AFB_1 at C4 (Carbon 4) or C22 and AFB_{2a} is formed in certain avian species as a result of hydration of the C2 - C3 double bond (Patterson & Roberts, 1970). AFP_1 results from o-demethylation of AFB_1 at C15, while aflatoxicol is the only metabolite of AFB_1 produced by a soluble cytoplasmic reductase enzyme system and the metabolism process occurs at C11 (Dhanasekaran *et al.*, 2011). The main chronic disease outcome associated with the intake of AFB_1 is hepatocellular carcinoma (HCC) or liver cancer. The disease is the third most common cause of cancer deaths globally (Ferlay *et al.*, 2010). The disease burden as a result of AF exposure remains significant, especially in many developing countries of Southeast Asia and

Sub-Saharan Africa. Studies of diversified adverse health effects of AFB_1 are very important topics in environmental health and toxicological research. AFs are known to cause human aflatoxicosis, the poisoning that results from acute exposure to AFs (Williams *et al*., 2004), and other health problems in humans and domestic livestock globally. Occurrence of acute aflatoxicosis can be a result of moderate to high level direct consumption of AFs. Some of the symptoms associated with this acute episode include hemorrhaging, acute liver damage, edema, alteration indigestion and possibly death (Yan Liu *et al*., 2012). A major outbreak of aflatoxicosis occurred in rural Kenya in April 2004 (Lewis *et al*., 2005). Epidemiologic investigations determined that the outbreak in which acute hepatotoxicity was identified among affected individuals was the result of AF poisoning from eating contaminated maize (corn). It was reported that 317 people were affected and 125 deaths were recorded during this outbreak (Lewis *et al*., 2005). Another notable episode of aflatoxicosis occurrence was recorded in western India in 1974. In that event, almost 400 people became ill with fever and jaundice while 106 deaths were recorded after eating contaminated maize (Reddy & Raghavender, 2007).

A previous review on AF exposure and its impacts on human populations stated that more than 4.5 billion people who live in developing countries around the world between the region of latitudes 40°N and 40°S were at risk of chronic exposure to largely uncontrolled amounts of AF (Williams *et al*., 2004). Unlike in developed countries such as the United States and European Union countries where methods to reduce AF contamination in food commodities are present and strict regulations are implemented and adhered to, developing countries cannot realistically achieve such minimal contamination strategies because of the characteristics of the food systems and the technological infrastructure in those countries (Williams *et al*., 2004). AFB_1 has the potential to affect the immunity of chronically exposed persons thereby leading to

susceptibility of affected individuals to other forms of diseases and even more tragic outcomes like death (Williams *et al*., 2004). AFB_1 exposure in animals have also been shown to affect certain immune parameters (Qian *et al*., 2014). The authors at the end of the latter study discovered that AFB_1 affected cell-mediated parameters, specifically by modulating both splenic lymphocyte phenotypes and cell-specific cytokine production in male F344 rats that were acutely and chronically exposed to AFB_1. A study of immunotoxicological effects of AFs in rats based on the immunological results showed a significant decrease in lymphocytes of the total white blood cells, immunoglobulin profile (Ig G and Ig A), T-cell subtypes ($CD3^+$, $CD4^+$ and $CD8^+$), NK cells, and pro-inflammatory cytokines (TNFα and IL-1β) which is typical of aflatoxicosis (Abbes *et al*., 2010). In many regions of the world, maize which is a very important source of mycotoxin exposure is a staple part of people's diet and AFB_1 is a common contaminant of maize and has been frequently found in high levels in Sub-Saharan Africa, Central America and Southeast Asia (Mitchell *et al*., 2014). Based on the data for consumption of staple foods acquired from the Food and Agriculture Organization (FAO) for 38 countries in Sub-Saharan Africa and the data for the 2004 mortality rates for all causes from these countries reported by the World Health Organization (WHO), results showed that there was significant correlation between consumption of maize and HIV-related deaths (Williams *et al*., 2010). Significant correlation between maize consumption and esophageal cancer was also reported (Williams *et al*., 2010). Previous epidemiological studies in human populations and animal studies have shown a strong correlation between AFB_1 exposure and liver cancer (Asim *et al*., 2011; Hamid *et al*., 2013; Jeannot *et al*., 2012). Chronic exposure to AFs in human populations is more common than acute toxic exposures (Jolly *et al*., 2008) and such chronic exposure to AFs is associated with HCC in humans, especially in those infected with hepatitis B virus (HBV) (Xu *et al*., 2010).

The HCC study was carried out in Southern Guangxi area of China where HCC is endemic and chronic HBV infection and dietary AF exposure are the major risk factors of HCC in the area. At the end of the study, additive effects of HBV basic core promoter mutations and high serum AFB_1-lysine adduct level was identified in the risk of developing HCC. Also, previous immune studies in both humans and animals have showed that exposure to varying levels of AFB_1 causes immune suppression (Bondy & Pestka, 2000; Jiang *et al*., 2005) and such lowering of immune system has been identified as a causal factor for impaired resistance to both infectious diseases and chronic infections (Williams *et al*., 2004). Using *in vitro* models, AFB_1 has been shown to impact upon various immune parameters and metabolic enzymes. Results of a study by Hanioka and colleagues in 2012 identified in HepG2 cells (a widely used human hepatocellular carcinoma cell line in immune studies) exposed to AFB_1 increased concentration of phase II enzymes, UDP-glucuronosyltransferase isoforms which suggest close contribution to toxicity of AFB_1 (Hanioka *et al*., 2012). Previous AFB_1 exposure studies using *in vitro* cellular models and animal models (Abbès *et al*., 2010; Lu *et al*., 2013) have identified the impact of AFB_1 on immune parameters. Abbes and colleagues in their immune study involving the exposure of AFB_1 and some other model genotoxins to human derived liver-cell lines and HepG2 cells investigated which of the cell lines or cells was more suitable to use in genotoxicity tests. Results showed that AFB_1 caused the most significant induction of DNA-migration in HepG2 cells followed by HCC1.2, a cell line isolated from primary human HCC and also in NKNT-3, a virally immortalized cell line derived from human hepatocytes (Winter *et al*., 2008). In an immune study in 2013, Lu and his colleagues employed the use of integrated analysis of general toxicity studies, transcriptomics and metabolomics to investigate the dose-dependent response in the liver tissue and serum from rats dosed with three levels of AFB_1 (0.25, 0.75 and 1.5mg/kg body weight) as acute exposure.

The study data suggested that AFB_1-induced acute hepatotoxicity may occur at higher doses based on general toxicity. Gene expression analysis showed that genes involved in the detoxification and oxidative stress responses were significantly induced by all AFB_1 treatment (Lu *et al*., 2013).

Reproductive toxicity of AFB_1

In animal studies, AFB_1 has been found to be a reproductive toxicant (Faridha, Faisal, & Akbarsha, 2006; Shuaib *et al*., 2010). Agnes and Akbarsha in their 2003 study identified reproductive effects such as decrease in sperm concentration and motility as well as an increase in abnormalities in the sperm resulting in decreased fertility in mice chronically exposed to AFB_1 i.e. 50 µg/kg body weight of AFB_1 for 7,15, 35 and 45 days (Agnes & Akbarsha, 2003). Female rats exposure to AFB_1 at a dose of 7.5 mg/kg body weight for 14 days identified significant deleterious effects on the ovary and uterus of the exposed animals as well as reductions in the conception rates and litter sizes, suggesting severe impairment of fertility in AFB_1-exposed rats compared to unexposed control animals (Ibeh & Saxena, 1997).

Studies in humans have shown that AFs crossed the human placental barrier and were internalized in the fetus (Lamplugh *et al*., 1988) and causing significant negative health effects in infants (Abdulrazzaq *et al*., 2004). Abdulrazzaq and colleagues at the end of their study observed a strong negative correlation between AF levels and birthweight of newborns. Another AF contamination study in humans identified high levels of various AF types (AFB_1, AFG_1 and AFQ_1) in human cord sera samples immediately after birth (Denning *et al*., 1990). Out of 35 sera samples collected, 17 (48%) of them contained AF with mean value 3.1 nmol/mL (range 0.064-13.9 nmol/mL) demonstrating transplacental transfer and concentration of AF by the feto-placental unit. A human population case control study conducted to assess the impact of AFB_1 on

human reproduction provided evidence suggesting the reproductive toxicity of AFB_1 among infertile men (cases) compared to control (fertile) men (Uriah, Ibeh, & Oluwafemi, 2001). At the end of the study, it was identified that the incidence of AFB_1 occurred in 11 (37%) out of 30 subjects who were infertile. Also, it was observed that more respondents in the infertile men group had higher concentrations of AFB_1 in their semen (range: 60 - 460 ng/ml) than in their serum (range: 170 - 350 ng/ml) compared to the control subjects whose semen AFB_1 level (range: 0 - 5 ng/ml) was lower compared to their serum AFB_1 level (5 - 20 ng/ml). The mean semen AF concentration level for the control subjects (0.5 ng/ml) was significantly lower level than those of the cases (350.77 ng/ml) ($p<0.05$), indicating that high AFB_1 exposure might be a contributory factor to infertility.

Developmental toxicity of AFB_1

AFB_1 has been identified in previous studies to cross the placental barrier and cause significant health defects to a growing fetus. Wangikar and colleagues in their 2004 study identified significant morphological malformations occurring in the offspring of Wistar rats exposed to 0.5 and 1 mg/kg dose of AFB_1 (Wangikar *et al*., 2004). At the end of the study, histological examinations of the fetal tissues showed that organs such as the liver, brain, kidney and to some extent the heart were negatively affected and the results showed that the extent of the various anomalies were largely proportional to the dose applied. In another animal study conducted earlier, Smith and Panciera identified that the fetuses of hamsters administered AF intraperitoneally at doses of 4 or 6 mg/kg body weight on days 8 and 9 of pregnancy experienced growth retardation compared with controls (Schmidt & Panciera, 1980).

Human population studies have also identified developmental effects of AFB_1 especially among children. In a prospective study conducted in Tanzania where maize is a staple diet of the

locals, consumption of AF-contaminated maize was associated with stunted growth among children (Shirima *et al*., 2015). At the end of the study, increasing growth impairment was observed among the children (6-14 months old at recruitment) as they increased in age with reduction of breastfeeding and increased complementary feeding. Increased complementary feeding meant that the children were fed more maize-based diets. The overall proportions of stunted children (length-for-age *z*-score below -2) were 44% at recruitment, 55% at 6 months from recruitment and 56% at 12 months from recruitment (Shirima *et al*., 2015). The authors concluded that exposure to fuminosin B_1 (another mycotoxin which has been confirmed to have negative impact on human health) alone or as a co-exposure with AFB_1 may have contributed to child growth impairment. A longitudinal study conducted in The Gambia demonstrated that maternal exposure to AF during pregnancy had a deleterious effect on the subsequent growth of infants (Turner *et al*., 2007). Maternal AF-albumin level was significantly related to height-for-age *z*-score (HAZ) in infants, such that HAZ decreased by about one fifth of a standard deviation for every increase in one unit of log average maternal AF-albumin level (Turner *et al*., 2007).

Transgenerational effects studies

Transgenerational effects studies are closely related to transgenerational inheritance studies in epigenetics. Such studies have been conducted using animal models such as rats and mice (Bruner-Tran & Osteen, 2011; Mannikam *et al*. 2012). Transgenerational inheritance is the basic mechanism that involves the ability of an environmental factor or compound to alter the germ line DNA methylation program to promote imprinted-like sites that then transfer an altered genome which subsequently promotes adult onset disease phenotypes transgenerationally (Skinner, Mannikam & Geurrerro-Bossagna, 2010). Thinking more about the long-term effects of environmental toxicants that people are exposed to everyday is necessary. This is because the

consequences as a result of such exposures should not only be a source of concern to individuals exposed and their children but also for the health of generations to come. Studies to investigate the potential epigenetic transgenerational impacts of a variety of different toxicants have also been conducted previously (Hunt *et al*., 2009, Mannikam *et al*., 2012). Transgenerational effects of chemicals have been studied previously using animal models. The majority of the toxicants that have been studied are endocrine disrupting chemicals (e.g. bisphenol A, dibutyl phthalate, VCZ, etc.) which are substances present in the environment and in food that interfere with the biosynthesis, metabolism and action of hormones (Stouder & Paoloni-Giacobino, 2010). Stouder and Paoloni-Giacobino discovered in their study that the reproductive effects of VCZ that occurred in the offspring (F_1 generation) of pregnant mice that were dosed with the toxicant were transgenerational. However, the effects gradually disappeared from F_1 to F_3 generation. Another transgenerational effects study conducted was by Andrea Cupp at the Washington State University. Her study investigated how exposure to the pesticide methoxychlor affected the sex determination of embryonic animals and revealed some important findings in the reproductive system of the offspring of exposed animals (Schmidt, 2013). Cupp and colleagues in their study discovered that the transient exposure of endocrine disruptors vinclozolin (VCZ) and methoxychlor induced an adult phenotype in the F_1 generation of decreased spermatogenic capacity and increased incidence of male infertility (Anway *et al*., 2005). The effects were transmitted through the male germline to subsequent generations (F_1 to F_4).

Limited studies have been done to investigate the transgenerational effects of mycotoxins in animal models. A transgenerational effect study in female pigs was previously conducted (Schoevers *et al*., 2015) with zearalenone (ZEN), a mycotoxin produced by various species of *Fusarium* (Li *et al*., 2015). It was observed from the study results that ZEN exposure caused a

reduction in the ovarian primordial follicle population of F_1 generation female pigs whose parents (i.e. F_0 generation animals) were exposed to ZEN during pregnancy and it was suggested that the effect caused may lead to a premature exhaustion of the follicle pool and risk of reduced litter sizes . Several studies to investigate the endocrine disrupting effects of mycotoxins have been carried out as well (Hueza *et al*., 2014; Storvik *et al*., 2011) with results indicating that the mycotoxins do impact upon endocrine receptors. In the latter study which was an *in vitro* study, JPEG cells (a human choriocarcinoma cell line) were exposed to sub-chronic levels of AFB_1. Results identified that AFB_1 which has been previously demonstrated to be transferred through the placenta (Denning *et al*., 1990) caused significant effects on genes important in endocrine regulation in placental cells.

Caenorhabditis elegans

The South African scientist, Sydney Brenner is regarded as the one who pioneered the active use of the nematode *Caenorhabditis elegans* (*C. elegans*) as a model organism for research in molecular and developmental biology (Brenner, 1974). *C. elegans* is a microbivore that has often been described to inhabit soil and leaf-litter environments in many parts of the world. The nematode has gained prominence over the years as a reliable model for *in vivo* toxicity studies. Some of the reasons the animal has become a significant model of choice include its cheap maintenance under laboratory conditions with the bacteria *Escherichia coli* (*E. coli*) OP50 usually serving as its source of food in liquid or on solid media. It is of relatively small size (adult ~1mm in length) and its shape like all other nematodes is an unsegmented, cylindrical body that tapers at both ends (Strange, 2006). Its transparent body at all stages of development allows for visualization of all cells by differential interference contrast and various forms of fluorescence microscopy. Its short generation cycle (~3 days) and lifespan (~3 weeks),

its sexual dimorphism with self-fertilizing hermaphrodites and cross-fertilizing males leading to large brood sizes of ~300 and ~1000 F_1 progeny are characteristics for serving as a good model organism for reproductive and developmental studies. The animal has a complete and invariant cell lineage map (from zygote to adult) with 959 and 1031 somatic cells of the adult hermaphrodite and male, respectively. Also, the successfully completed genome sequence of the nematode ~19,700 coding sequences and ~1300 noncoding RNAs, repositories of genomic and cDNA clones and a stock center for mutant animals and ongoing efforts to include null mutants for every gene as well makes the animal enjoy a diverse form of usage in various fields of research (Kampkötter *et al*., 2008). The similarity of the genome of the nematode to that of humans has also made it enjoy wide usage in areas of research including environmental toxicology and biomedical research (Kaletta & Hengartner, 2006; Leung *et al*., 2008) and depending on the bioinformatics approach used, a relatively high number of *C. elegans* homologues (60-80%) have been identified in human genes (Riddle *et al*., 1997) and 12 out of 17 known signal transduction pathways are evolutionarily conserved from *C. elegans* to humans (Brenner, 1974).

Previous studies have been carried out identifying growth, lethality, lifespan, reproduction and behavior as toxicological end points in research involving the use of *C.elegans* (Dhawan, Dusenbery, & Williams, 1999; Popham & Webster, 1979) and these studies also confirmed that these end points are quantifiable parameters of the health of the nematode. The purpose of choosing *C. elegans* as the model organism for the transgenerational study is based on the fact that *C. elegans* have a short generation time (life cycle at 20°C takes 3 days) (Table 1) and they are less expensive to conduct *in vivo* studies with compared to using other higher and established animal model systems. Also, the experimental potential presented by using *C.*

elegans for this study offers a system best suited for asking *in vivo* questions with relevance at the organismal level.

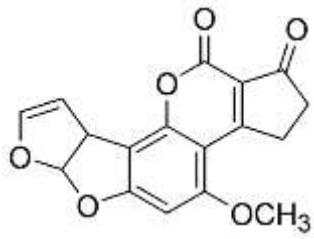

Figure 1: Chemical structure of AFB_1

Table 1: Lifecycle of *C. elegans* at different temperatures.

Temperature (^{0}C)	Embryogenesis (hours)	Molts (hours after hatch)				First eggs laid (hours after hatch)
		Larva 1 (L1)-L2	L2-L3	L3-L4	L4-adult	
16	29	24	39	53.5	74.5	94-97
20	18	15	24	34	46	59-60
25	14	11.5	18.5	26	35.5	45-46

Source: *Caenorhabditis elegans*; Modern Biological Analysis of an Organism (p. 9)

CHAPTER 2

INTRODUCTION TO PROJECT

A previous study aimed to investigate the probable transplacental transfer of AFs in humans (Denning *et al*., 1990) revealed that in the cord sera samples of 35 subjects enrolled, 17 (48%) of cord sera samples did contain AF, while out of the 35 maternal sera samples collected, only 2 contained AF, indicating a possible capability of AF to cross placental barrier and occurrence of concentrations at the feto-placental unit.

The toxic effects of AFB_1 has been studied extensively using various animal models. Qian and colleagues in their 2013 study used an integrative approach to assess the toxic effects of AFB_1 exposure on F344 rats (Qian *et al*., 2013). Several histological and biochemical parameters in the blood and liver that have been previously shown to get induced and also serve as good markers of AFB_1 exposure were reported. Serum aflatoxin B_1-lysine (AFB-lysine) adduct (an established biomarker of AFB_1 exposure) levels increased due to chronic exposure of the rats to AFB_1. Additionally, the results also showed that liver glutathione *S* transferases placental positive ($GST\text{-}P^+$) cells and foci which is used as a neoplastic marker in animal carcinogenesis models (Tatematsu *et al*., 1985; Sato, 1989) was induced by single and repeated exposures of AFB_1. A strong correlation between AFB-lysine adduct levels in serum and $GST\text{-}P^+$ cells and foci in rat liver samples exposed to single and repeated doses of AFB_1 was found in the study. Histological effect involving bile duct proliferation which is a characteristic of AFB_1-induced liver injury was also observed during the study.

The toxic effects of AFB_1 has been studied extensively in many *in vitro* and *in vivo* models (Pandey & Chauhan, 2007; Rastogi *et al*., 2006), and fewer using *C. elegans* (Leung *et al*., 2010; Yang *et al*., 2012). On the other hand, reproduction, lifespan, growth, behavior and assessment of oxidative damage are endpoints that have been shown to be important parameters when conducting toxicological studies in nematodes (Saul *et al*., 2014; Yu *et al*., 2011). All of these parameters are well studied and they can be used as quantifiable parameters of assessing adverse health effects of environmental toxicants in *C. elegans*. Brood size measures the ability of a toxin to prevent production of the next generation of animals while the growth assay which begins with the nematodes at the first larval stage (L1) and monitoring them over a period of 3 days allows to measure the toxic effects of a toxin on the L2, L3 and L4 developmental stages of the nematodes (Strange, 2006).

There are very limited information about the transgenerational effects of AFB_1 exposure, and therefore, we tried to take advantages of *C. elegans* model as described in Background and Literature Review to investigate the transgenerational effects of AFB_1 exposure.

The specific aims of this thesis study focused on:

- Assessing the toxicological impacts of sub-chronic exposure of AFB_1 on *C. elegans*, and
- Exploring the transgenerational effects of AFB_1 exposure from F_0 generation nematodes to transfer to F_1 and F_2 generation neonates.

CHAPTER 3

MATERIALS AND METHODS

C. elegans strains

The strains used in this study were the wild-type Bristol (N2) and RB864 or RB (nucleotide excision-repair deficient) ok698 strains of the nematodes. Nematodes were procured from the *Caenorhabditis* Genetics Center, Minneapolis, MN, USA.

Growth and collection of *C. elegans*

Both of the strains of *C. elegans* used in this study, the N2 and RB strains were cultured on nematode growth medium (NGM) solid agar plates seeded with *Escherichia coli* (*E. coli*) OP50 serving as food source and incubated at 20^0C as previously described (Brenner, 1974). The nematodes were monitored until they reached adult stage and they start laying eggs. The eggs were retrieved from agar plates and gravid nematodes were lysed with a bleaching mixture (2g NaOH dissolved in 40 mL of 5.25% NaOCl and 160 mL distilled water) to retrieve eggs that have not been laid from them. Age-synchronized L1-larvae or L4-larvae were collected as described previously (Donkin & Williams, 1995). Briefly, L1 nematodes were obtained after 24 hours of incubating eggs of the nematodes on NGM plates at room temperature while L4-stage worms were obtained after incubating the eggs of N2 and RB strain of the nematodes on NGM plates seeded with *E. coli* OP50 at 20°C for approximately 44 hours and 70 hours respectively.

Test Chemical

Stock solution of 10 mg/mL AFB_1 (AFB_1 purity: ≥98%, Sigma-Aldrich GmbH, Riedstr, Steinheim) was prepared in dimethyl sulfoxide (purity: ≥99%, Sigma-Aldrich GmbH, Riedstr, Steinheim) and stored in a glass bottle at -20^{0}C in the dark. This stock solution was used to prepare the test solutions on the days when the experiments were conducted.

Developmental toxicity experiment for F_0 generation

The development of the *C. elegans* was assessed by endpoint of growth which was determined by measuring the body length (µm). L1 stage nematodes were exposed to AFB_1. Briefly, gravid adult nematodes were cultured on nematode growth media (NGM) plates seeded with thin layer of *Escherichia coli* (*E. coli*) OP50 bacteria (the food of *C. elegans* on solid media). The plates were incubated at 20^{0}C. Eggs were collected from the plates by rinsing with M9 buffer into 15 mL centrifuge tubes. Gravid adults washed off the NGM plates along with the eggs were killed with bleach solution to ensure that only eggs are recovered after washing and centrifuging the eggs. The eggs were subsequently transferred to NGM plates with no food source and incubated at room temperature for 18-24 hours to get age-synchronized L1 stage nematodes. AFB_1 test solutions (Control or 0, 10, 30 and 90 µmol/L) were prepared and mixed with Lactose broth (L-broth) solutions before transferring them to sterile 12-well tissue culture plates. L-broth serves as food source for the nematodes in liquid-based experiments. Three replicate wells were used for each AFB_1 treatment group representing the three different time points (24, 48 and 72 hours) at which nematodes were assessed for change in body length. Subsequently, at least 20 individuals were added to each well containing AFB_1 test solutions and the plates were incubated at 20^{0}C to start exposure of *C. elegans* to AFB_1. On the first day of

exposing the nematodes to AFB_1, at least 20 L1 stage nematodes were retrieved separately and 2 drops of 10% formalin was applied to the solution on a watch glass containing the nematodes to immobilize them. Body length analysis was conducted with the aid of Olympus SXZ9 microscope (Olympus America Inc. Center Valley, PA, USA) and Infinity analyze software (V5.0.2, Lumenera Corporation, Ottawa, ON., Canada). The measurements recorded on the first day served as the baseline measurement for nematodes in each AFB_1 treatment group. After 24, 48 and 72 hours incubation, collection of 20 nematodes from each AFB_1 treatment group and capturing of their images as well as measurement of their body length was done in a similar manner as it was done on the first day of the experiment. Three independent growth experiments were carried out following the same protocol.

Reproductive toxicity experiments for F_0 generation

The reproductive toxicity was assessed using endpoints of brood size and generation time. To assess brood size of the F_0 generation nematodes (i.e. parental generation), six nematodes were examined per treatment. The experiment was carried out based on a procedure previously described (Swain *et al*., 2004). In brief, 6 L4-stage nematodes per AFB_1 treatment group were exposed in sterile 12-well tissue plates to 0, 10, 30 and 90 μmol/L AFB_1 test solutions mixed with L-broth. The plates were then incubated at 20^0C and the F_0 nematode was transferred to a new plate (replica-plated) containing only L-broth every 1.5 days. The transfer of the adults every 1.5 days was done over three cycles before terminating the experiment. Hatched progeny were counted the day following the transfer of the adult nematode.

Generation time (F_0 egg to first F_1 egg) was determined by visual inspection of the plates using ten adult nematodes per AFB_1 treatment and control groups for statistical purposes. The generation time experiment was performed by modifying a previous procedure (Venkateswara

Rao *et al*., 2006). Briefly, generation time in the F_0 generation was evaluated by exposing ten L4-stage nematodes per AFB_1-treatment group were exposed in sterile 12-well tissue culture plates to 0, 10, 30 and 90 μmol/L AFB_1 test solutions mixed with L-broth and the plates were incubated at 20^0C. The adults were transferred to fresh sterile 12-well tissue culture plates after exposing them to AFB_1 treatment for 1.5 days and then the plates were inspected after they have reproduced their first set of offspring. Nematodes present in each well after the time of growth were then counted to determine the generation time for each AFB_1 treatment group.

Model for determining the Generation time

Number of generations (n) = (log N_1- log N_0)/ log 2

N_1= number of nematodes in each well after time of growth

N_0= number of nematodes on day 0, that is number of nematodes initially exposed to AFB_1 treatment (10 per each treatment group)

Generation time (h) = time of growth/ number of generations

Developmental toxicity experiments for F_1 and F_2 generation

To investigate whether AFB_1 exposure to F_0 generation *C. elegans* has probable transgenerational effects on F_1 and F_2 generation nematodes, the endpoints of growth, brood size and generation time were evaluated for this purpose. The F_1 generation growth experiment was carried out by exposing F_0 generation nematodes to AFB_1 on solid media. L1 stage nematodes were exposed to AFB_1 dissolved in solid media and the eggs of the nematodes retrieved represented F_1 generation *C. elegans* which were subsequently used for this phase of experiment. Briefly, NGM was prepared and poured (12ml per plate) in 60 mm X 15 mm petri dishes which were subsequently stored at 4^0C until time for addition of AFB_1 test solutions. AFB_1 test solutions were prepared as previously described in the first phase of the experiment

and 1 mL of the different AFB_1 solutions were added to the NGM plates prepared earlier. The solutions were allowed to dissolve into the agar under sterile conditions in a laminar flow hood. Four NGM plates were used per each AFB_1 treatment group. After the AFB_1 test solutions had dissolved completely in the media, each plates was then seeded with a thin layer of the bacteria *E. coli* OP50 to serve as food source. The plates were subsequently incubated at 37^0C overnight to allow for the growth of the bacteria on the plates. The next day after bacterial growth has been observed on all the plates, eggs of *C. elegans* were collected from NGM plates seeded with *E. coli* OP50 which already contains adults laying eggs. The eggs were subsequently transferred to NGM plates containing the different AFB_1 test solutions dissolved in them and *E. coli* OP50. The plates were subsequently incubated at 20^0C. The nematodes that hatched out during this period serve as the F_0 generation nematodes that were exposed to AFB_1 and their offspring represent the F_1 generation nematodes. The nematodes belonging to each AFB_1-treated group were monitored until they reached adult stage and laid eggs. The eggs were subsequently transferred to NGM plates without food source and incubated at room temperature for at least 18 hours, maximum 24 hours to get age-synchronized L1 stage F_1 generation *C. elegans*. After incubation, at least 100 L1 stage nematodes were collected from each of the four plates representing each AFB_1 treatment group and at least 20 nematodes were collected from each AFB_1 treatment group separately. The images of twenty nematodes per AFB_1-treatment group were captured and their body length were measured and recorded as previously described in the F_0 generation growth experiment to serve as 0 hour (baseline) measurement for each group. Then at least 20 nematodes were added to each well of a sterile 12-well tissue culture plates containing L-broth only (1 mL per well). Three replicate wells were used for each AFB_1 treatment group representing the different time points of interest (24, 48 and 72 hours) when the body length of

the nematodes would be assessed. The plate was incubated at 20^0C throughout the three day period of the experiment. At least 20 nematodes were retrieved from each well of the AFB_1 treatment group after 24, 48 and 72 hours. Body length analysis was done following the same procedure used in the F_0 generation growth experiment.

For the F_2 generation growth experiment, we retrieved the offspring of F_1 generation worms for this phase of the experiment. F_1 nematodes were incubated on NGM plates seeded with *E. coli* OP50 at 20^0C and monitored until they reached adult stage and laid eggs. The eggs were subsequently retrieved and incubated on NGM plates without food source and incubated at room temperature for at least 18 hours to collect L1 stage F_2 generation nematodes. The L1 stage nematodes were subsequently washed off the NGM plates and similar to how it was done in the F_1 generation growth experiment, at least 100 representative nematodes were retrieved from each AFB_1 treatment group. At least 20 L1 stage nematodes from each AFB_1 treatment group were collected separately on the first day of exposure and their images were captured and their body length was measured and recorded to serve as 0 hour measurements. At least twenty nematodes were subsequently transferred to each well of a sterile 12-well tissue culture plate containing 1 mL of L-broth solution. Three wells were used for each AFB_1 treatment group representing the different time points of interest (24, 48 and 72 hours) when the body length of the nematodes were assessed. We retrieved at least 20 nematodes from each well of the AFB_1 treatment groups after 24, 48 and 72 hours. Body length analysis was done following the same procedure used in the F_0 and F_1 generation growth experiments.

Reproductive toxicity experiments for F_1 and F_2 generation

For the F_1 generation brood size experiment, we retrieved the progeny of the F_0 generation nematodes. Six L4 stage nematodes representing each AFB_1 treatment group and control were collected and then exposed to L-broth only in fresh sterile 12-well tissue culture plates. The plates were subsequently incubated at 20^0C and the F_1 adult nematodes were transferred to new 12-well plate containing only L-broth every 1.5 days. The transfer of the adult nematodes every 1.5 days was done over three cycles until the experiment was terminated. Hatched progeny were counted the day following the transfer of the F_1 nematode. The F_2 generation brood size experiment was carried out following the same procedure we used in the F_1 generation brood size experiment and we retrieved F_2 generation nematodes as offspring from the F_1 generation nematodes.

In the generation time experiment conducted for F_1 generation nematodes, 10 neonates of the F_0 generation nematodes were retrieved to ensure significant results were achieved. They were transferred to new sterile 12-well tissue culture plates containing only L-broth. Two nematodes were incubated in five wells per AFB_1 treatment group and once they reached L4 stage they were transferred to a new sterile 12-well tissue culture plate and monitored for 1.5 days. By the end of this period, nematodes present in each well were then counted to determine the generation time for the representatives of each AFB_1 treatment group.

The generation time experiment in the F_2 generation was conducted following the same procedure used in the generation time experiment carried out in F_1 generation. The offspring of the F_1 generation nematodes were retrieved and used for this phase of experiment.

Statistical Analyses

EC_{50} values and their 95% Confidence Intervals in the phase 1 and phase 2 sections of the study were calculated using PROBIT procedure. Homogeneity of variance and normality of errors assumptions were checked using Shapiro–Wilk's test. A Generalized Linear Model was used to evaluate the significant difference among AFB_1 treatment groups and between all treatment levels and the control group in the first phase of the experiment (F_0 generation). Levels of 0.05 was considered statistically significant. The ratio of the EC_{50} of parent nematodes to that of the offspring was calculated using the F_1 and F_2 generation experiments results. A Mixed-effects model based on time, dose and time x dose was calculated using the F_1 and F_2 generation data. All statistical analyses were performed using GraphPad Prism 6.0 version (GraphPad Software Inc. San Diego, California, USA) and IBM SPSS 23 version (IBM Corp. Armonk, NewYork, USA) statistical software packages.

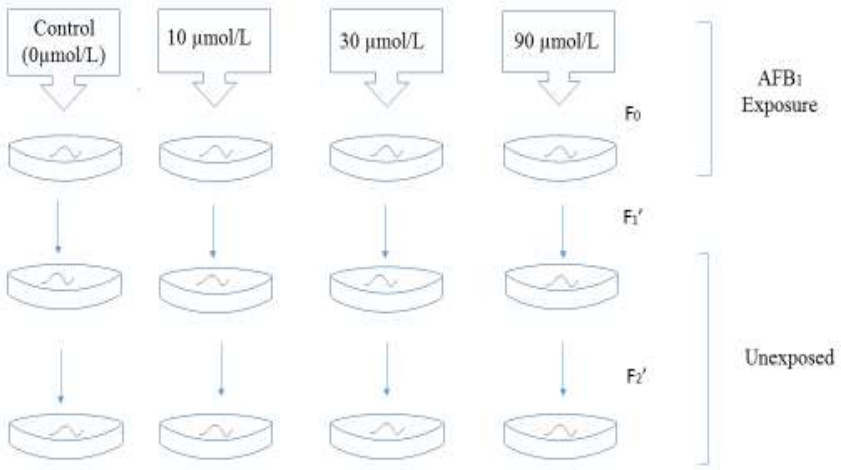

Figure 2: Flow chart for study design. F_0 generation nematodes are exposed to the AFB_1 treatment solutions and monitored for reproductive and developmental effects while F_1 and F_2 generation nematodes were unexposed to AFB_1 and they are also monitored to identify for likely transgenerational effects in their reproduction and development.

CHAPTER 4

RESULTS

Effect of AFB_1 exposure on growth in F_0 *C. elegans*

The growth experiments showed that the mean body length of the nematodes in the AFB_1-treated groups decreased in a concentration-dependent manner at each time point of measurement compared to nematodes in the control group (Figures 3&4). After 24 hours of AFB_1 exposure, mean body length of the nematodes in the 10, 30 and 90 µmol/L treatment was significantly decreased by 21%, 31% and 50%, respectively, in the N2 *C. elegans*, as compared to the control group. After 48 hours and 72 hours AFB_1 exposure, a similar concentration-dependent trend was observed in body length of N2 *C. elegans* as it decreased by 34%, 53% and 72%, respectively, in 10, 30 and 90 µmol/L AFB_1 treatment groups after 48 hours, and by 20%, 39% and 65%, respectively, in 10, 30 and 90 µmol/L AFB_1 treatment groups after 72 hours as compared to nematodes in the control group. The greatest growth impairment effect at each time point of measurement was observed in the highest concentration tested (90 µmol/L) (Figure 3). A similar trend was observed in the RB *C. elegans* (Figure 4). After 24 hours of AFB_1 exposure, the mean body length of the RB *C. elegans* in the 10, 30 and 90 µmol/L AFB_1-treatment groups decreased by 33%, 40% and 53%, respectively, as compared to the control group. After 48 hours exposure, the mean body length of the RB nematodes in the 10, 30 and 90 µmol/L AFB_1-treated groups decreased by 33%, 58% and 73%, respectively, as compared to the control group. A similar concentration-dependent trend was also observed after 72 hours exposure to AFB_1 with

mean body length of the RB *C. elegans* as the 10, 30 and 90 µmol/L AFB_1-treated groups decreased by 48%, 75% and 85%, respectively, as compared to the control group.

Effect of AFB_1 exposure on reproduction in F_0 *C. elegans*

From the reproduction experiments conducted, it was observed that AFB_1 caused reproductive impairment in the nematodes as demonstrated by significant reduction of brood size and extended generation time. The mean brood size of N2 *C. elegans* was reduced by 47%, 89% and 91.6% in the 10, 30 and 90 µmol/L AFB_1-treated groups, respectively, as compared to the control (0 µmol/L) group (Figure 5). A similar concentration-dependent trend was observed in brood size experiments with the RB *C. elegans* where there was 60%, 72% and 96% reduction in mean brood size of nematodes in the 10, 30 and 90 µmol/L AFB_1-treated groups, respectively, as compared to the control group (Figure 6).

Mean generation time in the N2 *C. elegans* showed a significant 2-fold increase ($p<0.001$) in the highest AFB_1 treatment (i.e. 90 µmol/L) group compared to the control group. The mean generation time in the 10 and 30 µmol/L AFB_1-treated groups were not significantly increased when compared to the control group (Figure 7). In the RB *C. elegans*, the mean generation time was not significantly extended in the 10 µmol/L AFB_1-treated group compared to the control group. However, the mean generation time of nematodes in the 30 and 90 µmol/L AFB_1-treated groups showed a significant 4-fold and 6-fold increase ($p<0.0001$), respectively, as compared to the control group (Figure 8).

Effects of AFB_1 on growth in F_1 *C. elegans*

As it was illustrated in the flow chart of the study design (Figure 2), the F_1 generation nematodes were not exposed to AFB_1 and hence were only assessed for the endpoints of growth, generation time and brood size to evaluate potential transgenerational effects of AFB_1 exposure

that occurred in the F_0 generation. The effect of AFB_1 on growth of nematodes that was observed in the F_0 generation experiment was present in the F_1 generation nematodes as well (Figure 9). F_1 generation results in the N2 *C. elegans* showed that at 0 hour (i.e. when the nematodes were at L1 stage) and after 24 hours, there was significant difference in the mean body length of nematodes belonging to the 90 µmol/L AFB_1-treated group compared to the control group. At 0 hour, the mean body length of nematodes in the 90 µmol/L AFB_1-treated group showed a 14% decrease compared to those in the control group ($p < 0.05$). While after 24 hours, the mean body length of nematodes in the 90 µmol/L AFB_1-treated group showed a 9% decrease compared to those in the control group ($p < 0.05$). The mean body length of nematodes in the 10 and 30 µmol/L AFB_1-treated groups did not show statistically significant difference as compared to those in the control group at 0 hour and after 24 hours. However, the mean body length of nematodes in the 10, 30 and 90 µmol/L AFB_1-treated groups decreased significantly as compared to the control group after 48 and 72 hours. Nematodes in the 10, 30 and 90 µmol/L AFB_1-treated groups decreased in mean body length by 7.2%, 9.7% and 15.4% respectively compared to the ones in the control group. Similar trend of decrease in mean body length was observed after 72 hours. Nematodes in the 10, 30 and 90 µmol/L AFB_1-treated groups decreased in mean body length by 6.6%, 8.9% and 14.2% respectively compared to those in the control group after 72 hours exposure to L-broth only. Although the decrease in mean body length that was observed in the F_1 generation nematodes were not as highly significant as was observed in the F_0 generation experiment, our results from the F_1 generation growth experiment still indicates that the effect of AFB_1 exposure that was present in the F_0 generation was transferred to the neonates of AFB_1-exposed nematodes thereby affecting their growth. Additionally, the trend of decrease in body length according to the different AFB_1-treatment groups observed in the F_1

generation nematodes was similar to the one observed in the F_0 generation as it was a concentration-dependent trend.

Effect of AFB_1 on reproduction in F_1 *C. elegans*

We observed from the results of the mean brood size in the F_1 generation of the N2 *C. elegans* that the mean brood size of nematodes belonging to the 10 and 30 µmol/L AFB_1-treated groups did not show statistically significant difference as compared to the control group ($p > 0.05$). However, the mean brood size of nematodes in the 90 µmol/L AFB_1-treated group decreased significantly by 42.3% as compared to the control group ($p < 0.05$) (Figure 10). From the results of RB *C. elegans*, the mean brood size of F_1 generation nematodes in the 10 µmol/L AFB_1-treated groups decreased by 70% compared to the control group nematodes. Mean brood size of F_1 generation nematodes in the 30 and 90 µmol/L AFB_1-treated groups decreased by 48% and 97% respectively compared to nematodes in the control group (Figure 11). The irregular trend of results observed in the RB *C. elegans* results among the AFB_1-treated groups could be as a result of experimental error.

In the generation time results for the F_1 generation of N2 *C. elegans*, only the nematodes belonging to the 10 µmol/L AFB_1-treated group showed a significant 2-fold increase ($p < 0.05$) in mean generation time as compared to the control group. The mean generation time of the nematodes in the 30 and 90 µmol/L AFB_1-treated groups did not show a statistically significant increase ($p > 0.05$) as compared to the control group (Figure 12). Results from the generation time experiment for the F_1 generation of RB *C. elegans* showed that nematodes belonging to the 90 µmol/L AFB_1-treated group had a significant 4-fold increase ($p<0.05$) in mean generation time as compared to the control group. The mean generation time of nematodes in 10 and 30

μmol/L AFB_1 treatment groups compared to the control group was not statistically significant (p=0.114) (Figure 13).

Effect of AFB_1 on growth in F_2 *C. elegans*

The results of the growth experiment carried out using F_2 generation N2 *C. elegans* suggests that the effect of AFB_1 exposure that was highly significant and concentration-dependent in F_0 generation and modestly significant as well in the F_1 generation nematodes became less significant in the F_2 generation. There was no clear concentration-dependent trend in the mean body length based on AFB_1-treated groups (Figure 14). However, the mean body length of the nematodes in the AFB_1-treated groups were still significantly decreased compared to the control group at the different time points of measurement especially in the 30 and 90 μmol/L AFB_1-treated groups. At the 0 hour time point (i.e. when the nematodes were still at L1 stage), the mean body length of worms in the 10, 30 and 90 μmol/L AFB_1-treated groups decreased by 14%, 28% and 15% respectively compared to the control group. After 24 hours, the mean body length of nematodes in the 30 and 90 μmol/L AFB_1-treated groups decreased by 14% and 2.4% compared to the control group nematodes. While the mean body length of nematodes in the 10 μmol/L AFB_1-treated group was higher than that of the control group after 24 hours. The mean body length of nematodes in the 10, 30 and 90 μmol/L AFB_1-treated groups decreased by 2.4%, 13% and 16% respectively compared to the control group after 48 hours exposure to L-broth only, while their mean body length decreased by 4.5%, 22% and 11% respectively after 72 hours exposure to L-broth only.

Effect of AFB_1 on reproduction in F_2 *C. elegans*

The mean brood size results of the F_2 generation in the N2 *C. elegans* did not show a clear concentration-dependent trend. Nematodes belonging to the 10 μmol/L AFB_1-treated group

had a higher mean brood size as compared to the control group while nematodes belonging to the 30 and 90 µmol/L AFB_1-treated groups had a 13.8% and 5.8% decrease in mean brood size as compared to the control group (Figure 15). All of these differences observed were not statistically significant ($p > 0.05$). An interesting observation was identified in the F_2 generation brood size experiment of our study using the RB strain of *C. elegans*. The results showed that effects of AFB_1 exposure on brood size of nematodes in the F_0 generation which was concentration-dependent was transmitted to F_2 *C. elegans* (Figure 16). The mean brood size of nematodes in the 30 and 90 µmol/L AFB_1-treated groups decreased by 33% and 97% respectively as compared to the control group. The 7% decrease in brood size observed in the 10 µmol/L AFB_1-treated group as compared to the control group was not statistically significant ($p > 0.05$).

In the F_2 generation results for the mean generation time of N2 *C. elegans*, we observed that mean generation time of nematodes belonging to the 10 and 90 µmol/L AFB_1-treated groups were not significantly increased as compared to the control group ($p > 0.05$). However, the mean generation time of the nematodes in the 30 µmol/L AFB_1-treated group showed a significant 2-fold increase ($p < 0.05$) as compared to the control group (Figure 17). The mean generation time results in the F_2 generation of RB *C. elegans* indicated that there were no statistically significant differences between nematodes in the AFB_1-treated groups as compared to the control group ($p > 0.05$) (Figure 18).

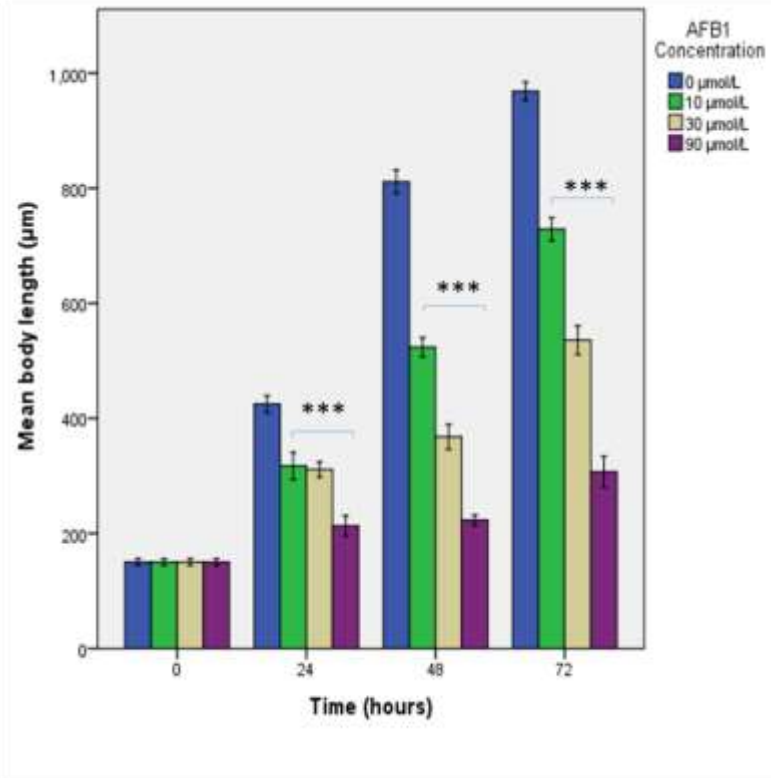

Figure 3: Effect of AFB_1 exposure on the growth of F_0 generation of *C. elegans* (N2 strain). Each bar represents the mean ± SE of three independent experiments and within each experiment body length of 20 nematodes per AFB_1 treatment group were measured at the time points of interest. ***= $p<0.0001$, compared to control.

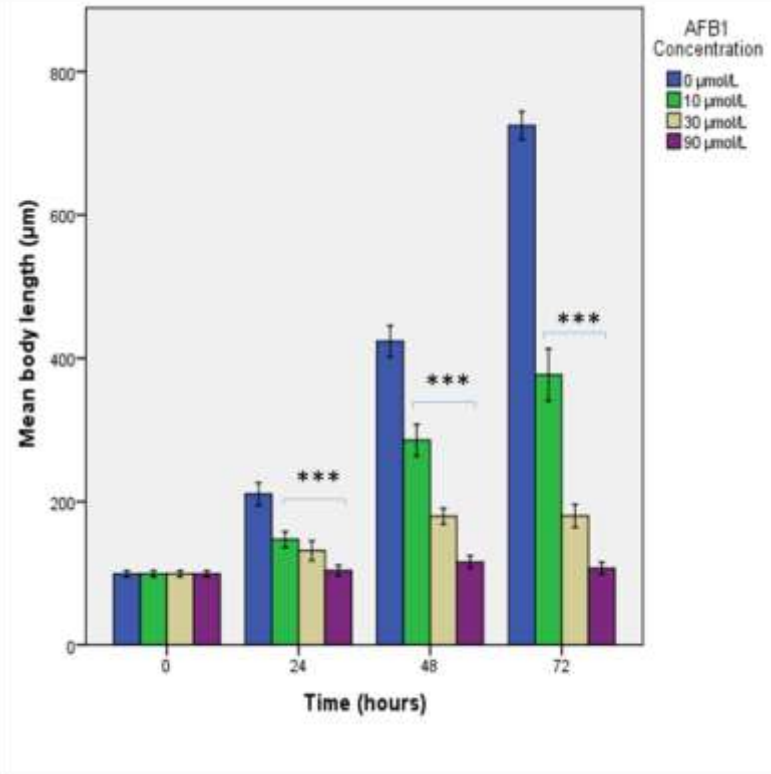

Figure 4: Effect of AFB_1 exposure on the growth of F_0 generation of *C. elegans* (RB strain). Each bar represents the mean body length ± SE of 20 nematodes at each time point of measurement. ***= $p<0.0001$, compared to control.

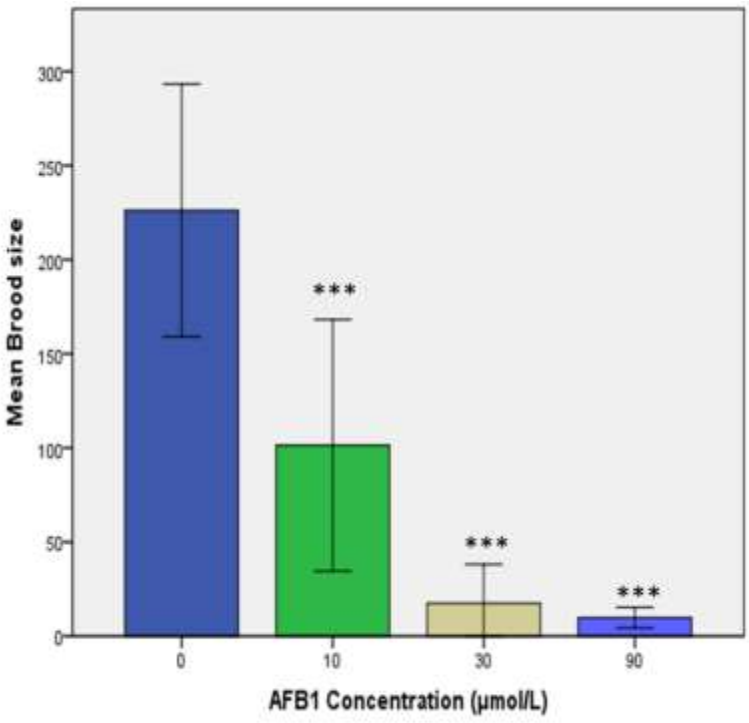

Figure 5: Effect of AFB_1 exposure on the brood size of F_0 generation of *C. elegans* (N2 strain). Each bar represents the mean ± SE of two independent experiments and 6 nematodes were exposed to AFB_1 in each group during both experiments. ***= $p<0.0001$, compared to control.

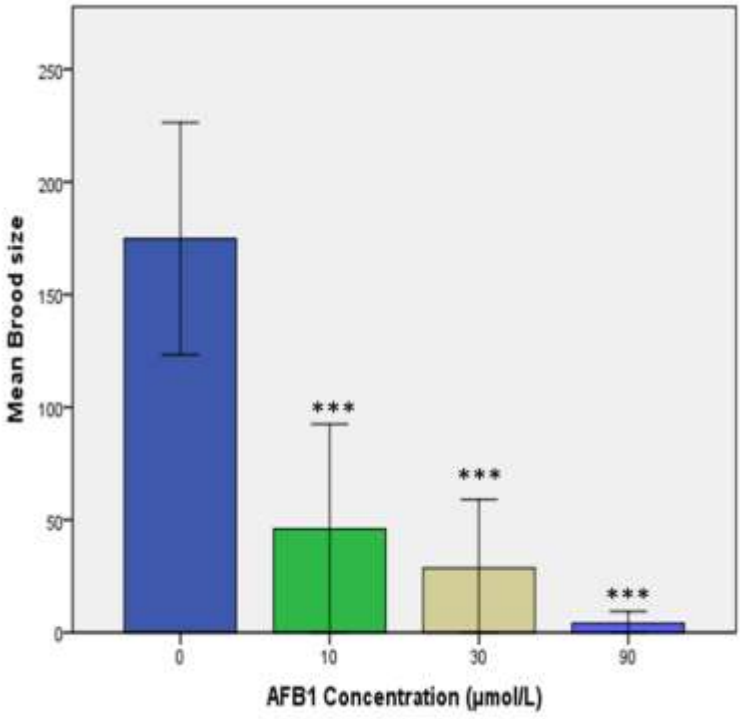

Figure 6: Effect of AFB_1 exposure on the brood size of F_0 generation of *C. elegans* (RB strain). Each bar represents the mean± SE of two independent experiments and 6 nematodes were exposed to AFB_1 in each group during both experiments. ***= $p<0.0001$, compared to control.

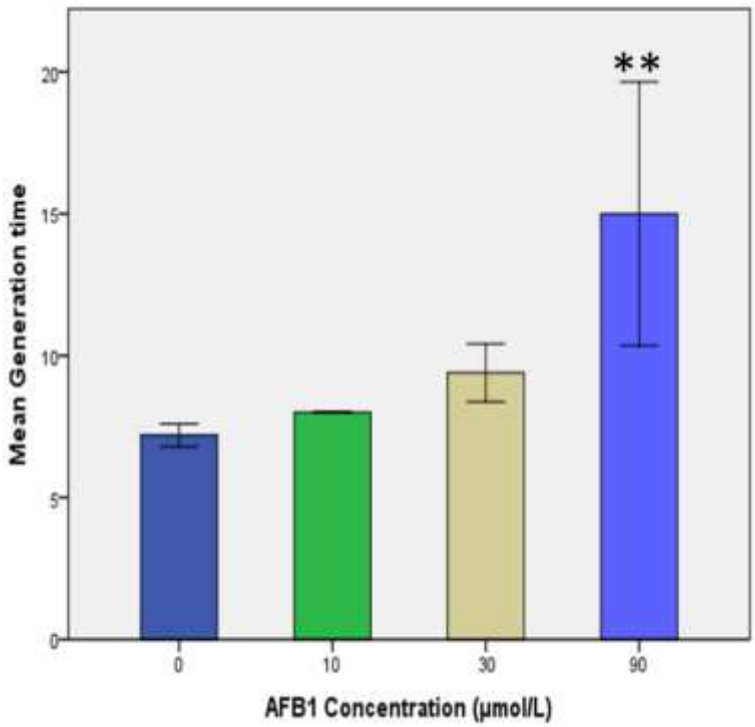

Figure 7: Effect of AFB_1 exposure on generation time in F_0 generation of *C. elegans* (N2 strain). Each bar represents the mean ±SE of two independent experiments and the mean generation time for 10 adult nematodes per AFB_1 treatment group was calculated during each experiment.
**= $p<0.001$, compared to control.

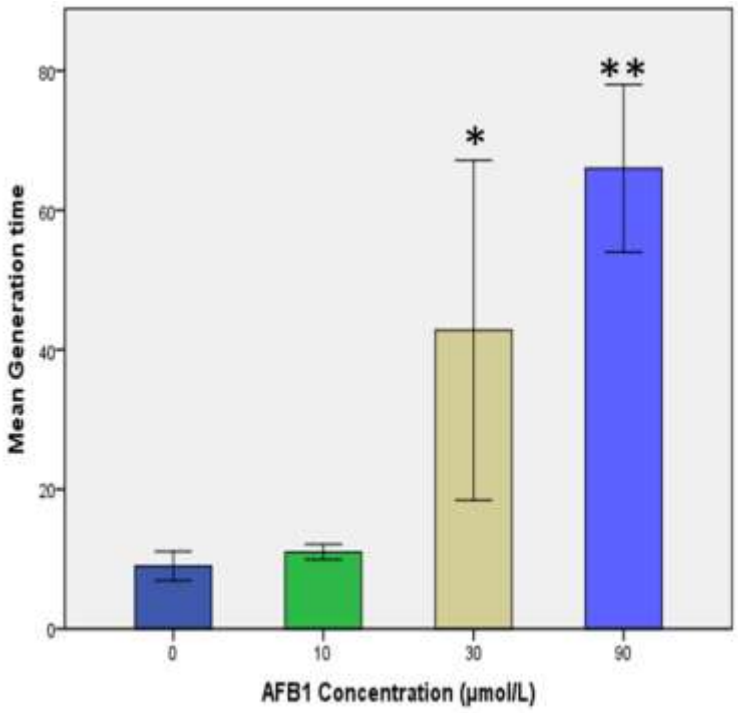

Figure 8: Effect of AFB_1 exposure on generation time in F_0 generation of *C. elegans* (RB strain). Each bar represents the mean generation time ± SE for 10 adult nematodes per AFB_1 treatment group. **= $p<0.001$ and *= $p<0.05$, compared to control.

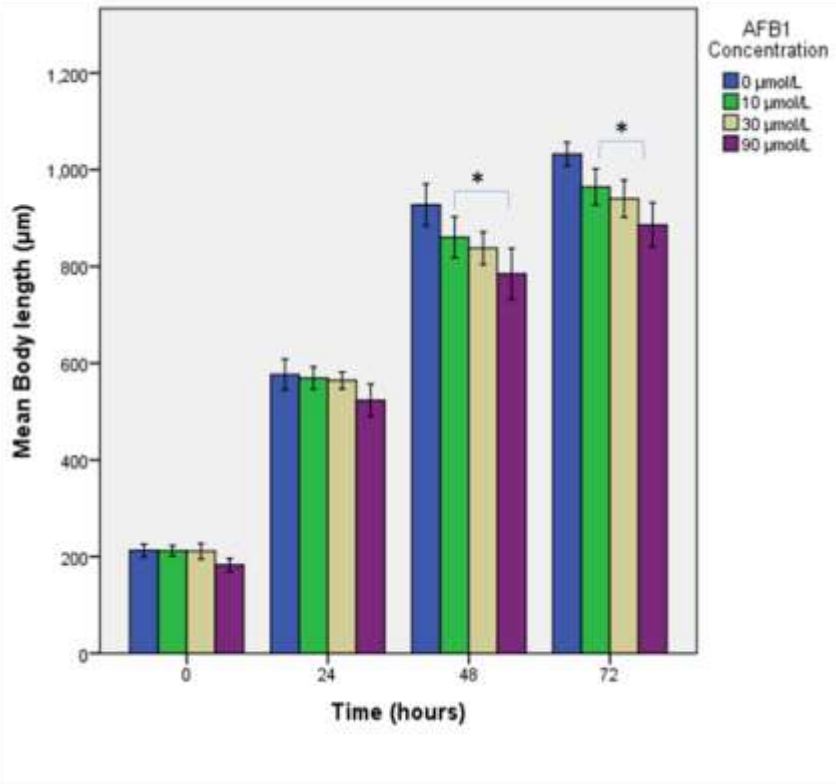

Figure 9: Effect of AFB_1 on growth in F_1 generation of *C. elegans* (N2 strain). Each bar represents the mean body length ± SE of 20 nematodes at each time point of measurement. *= $p<0.05$, compared to control.

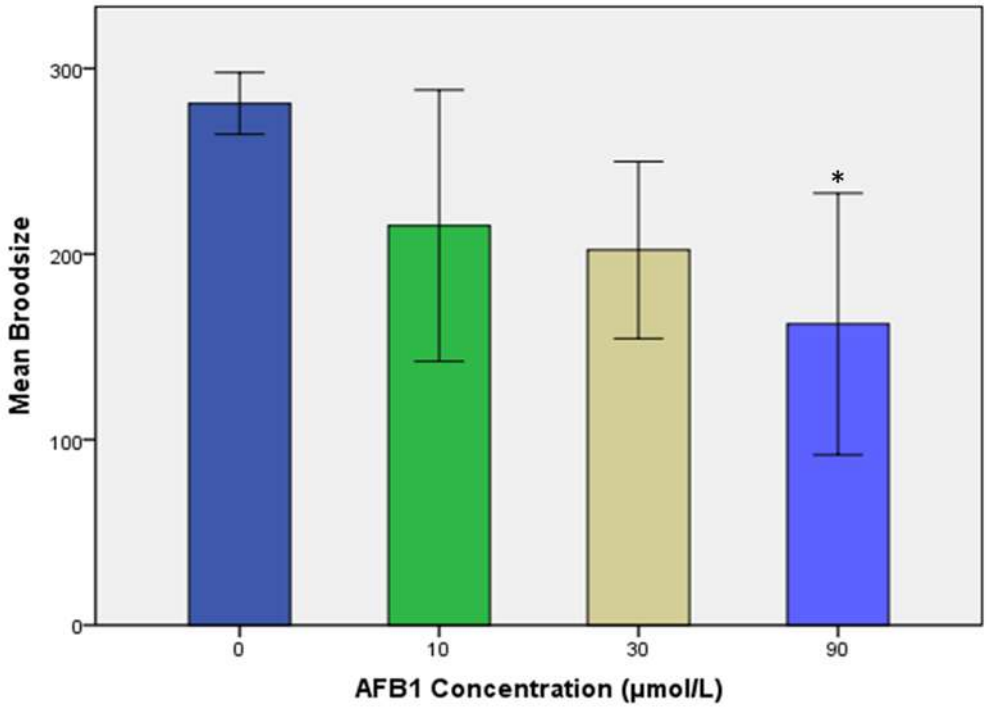

Figure 10: Effect of AFB_1 on the brood size in F_1 generation of *C. elegans* (N2 strain). Each bar represents the mean brood size ± SE of 12 adult nematodes per AFB_1 treatment group from two independent experiments. *= $p<0.05$, compared to control.

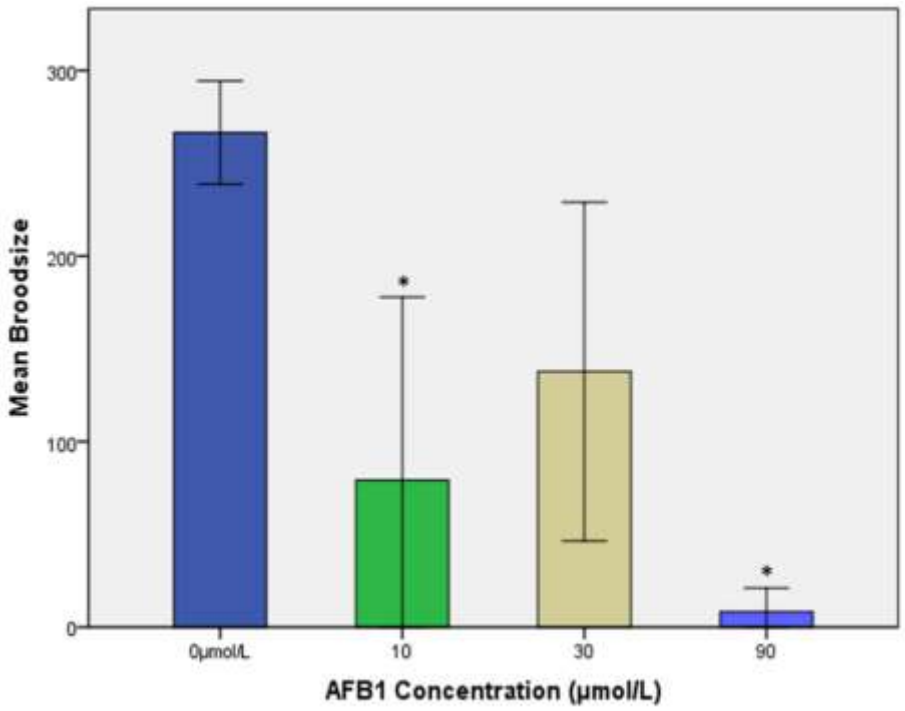

Figure 11: Effect of AFB_1 on the brood size in F_1 generation of *C. elegans* (RB strain). Each bar represents the mean brood size ± SE of 6 adult nematodes per AFB_1 treatment group.
*= $p<0.05$, compared to control.

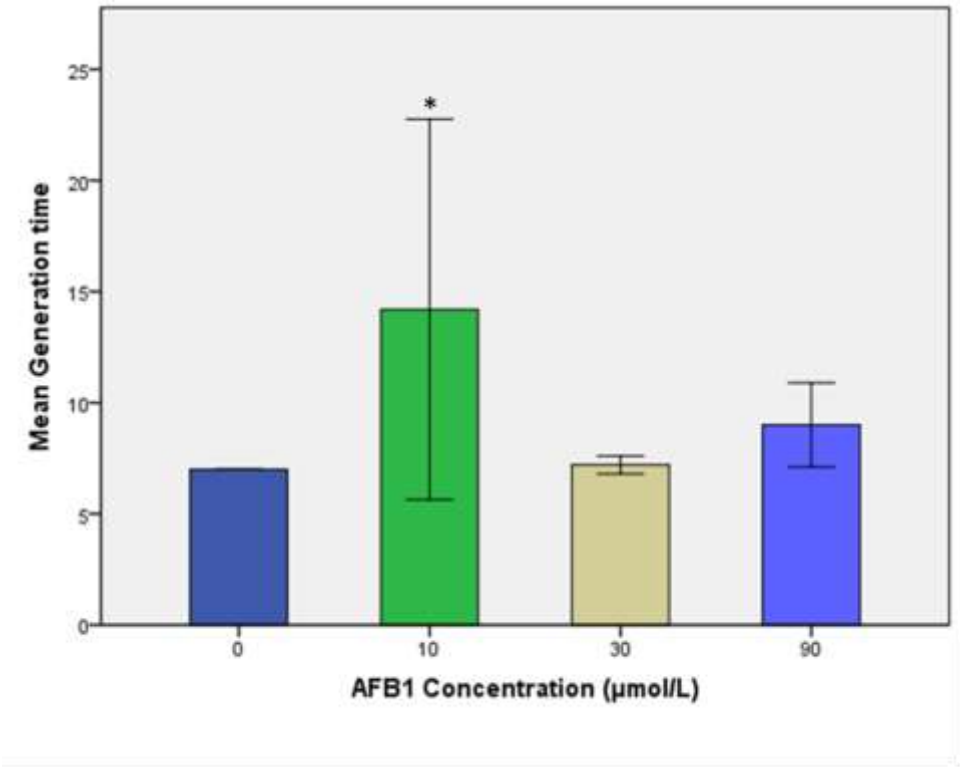

Figure 12: Effect of AFB_1 on generation time in F_1 generation of *C. elegans* (N2 strain). Each bar represents the mean generation time ± SE for 10 adult nematodes representing each AFB_1 treatment group. *= $p<0.05$, compared to control.

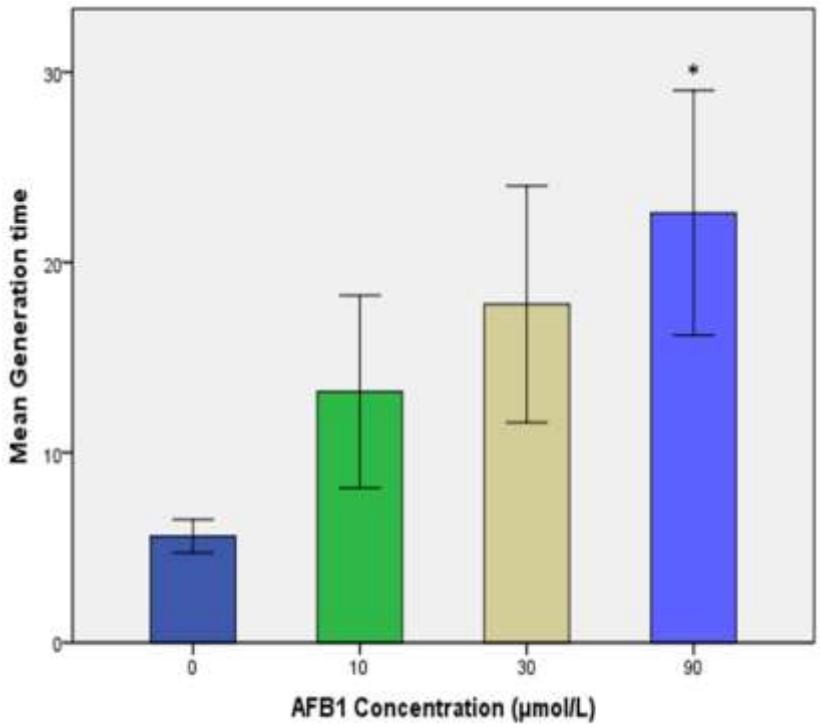

Figure 13: Effect of AFB_1 on generation time in F_1 generation of *C. elegans* (RB strain). Each bar represents the mean generation time ± SE for 10 adult nematodes representing each AFB_1 treatment group. *= $p<0.05$, compared to control.

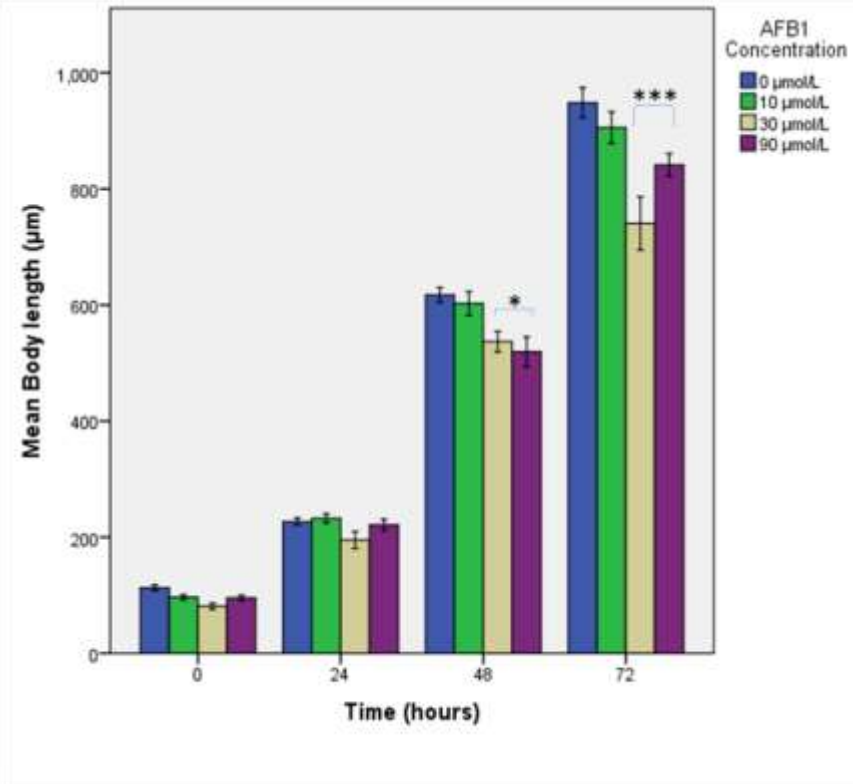

Figure 14: Effect of AFB_1 on growth in F_2 generation of *C. elegans* (N2 strain). Each bar represents the mean body length ± SE of 20 nematodes at each time point of measurement. *= $p<0.05$ and ***= $p<0.0001$, compared to control.

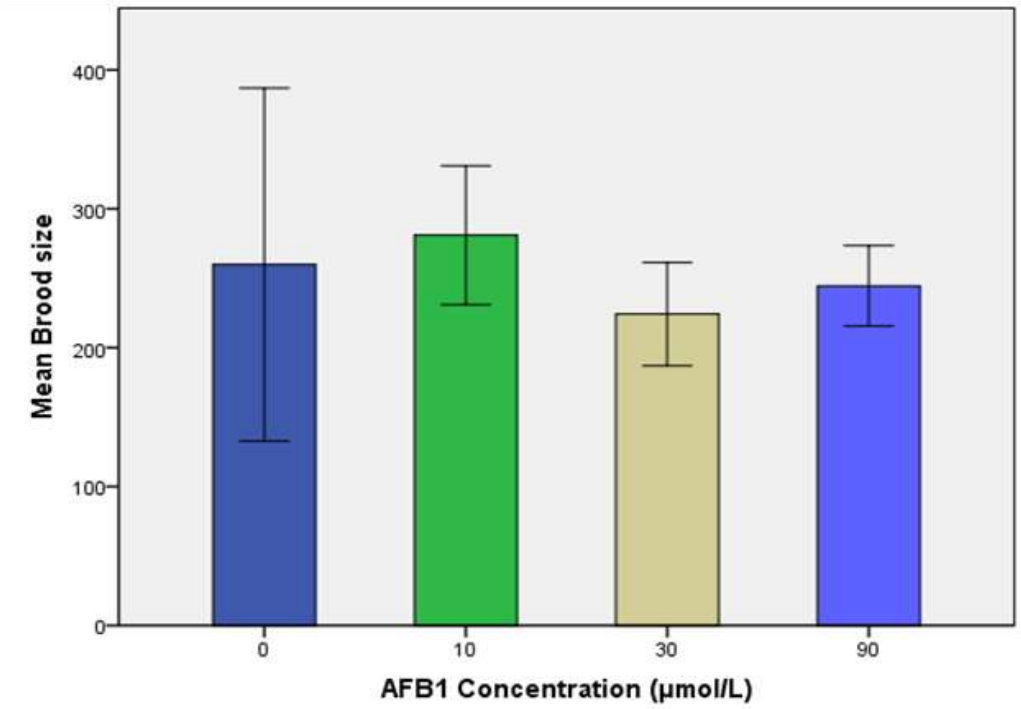

Figure 15: Effect of AFB_1 on the brood size in F_2 generation of *C. elegans* (N2 strain). Each bar represents the mean brood size ± SE of 6 adult nematodes per AFB_1 treatment group and no statistical significance was found ($p > 0.05$).

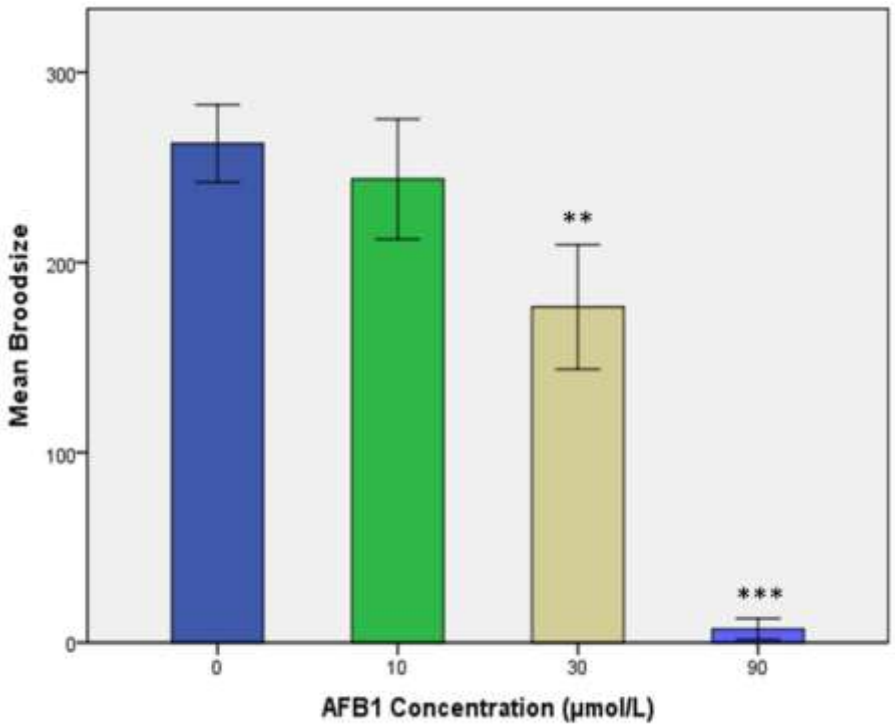

Figure 16: Effect of AFB_1 on the brood size in F_2 generation of *C. elegans* (RB strain). Each bar represents the mean brood size ± SE of 6 adult nematodes per AFB_1 treatment group.
**= $p<0.001$ and ***= $p<0.0001$, compared to control.

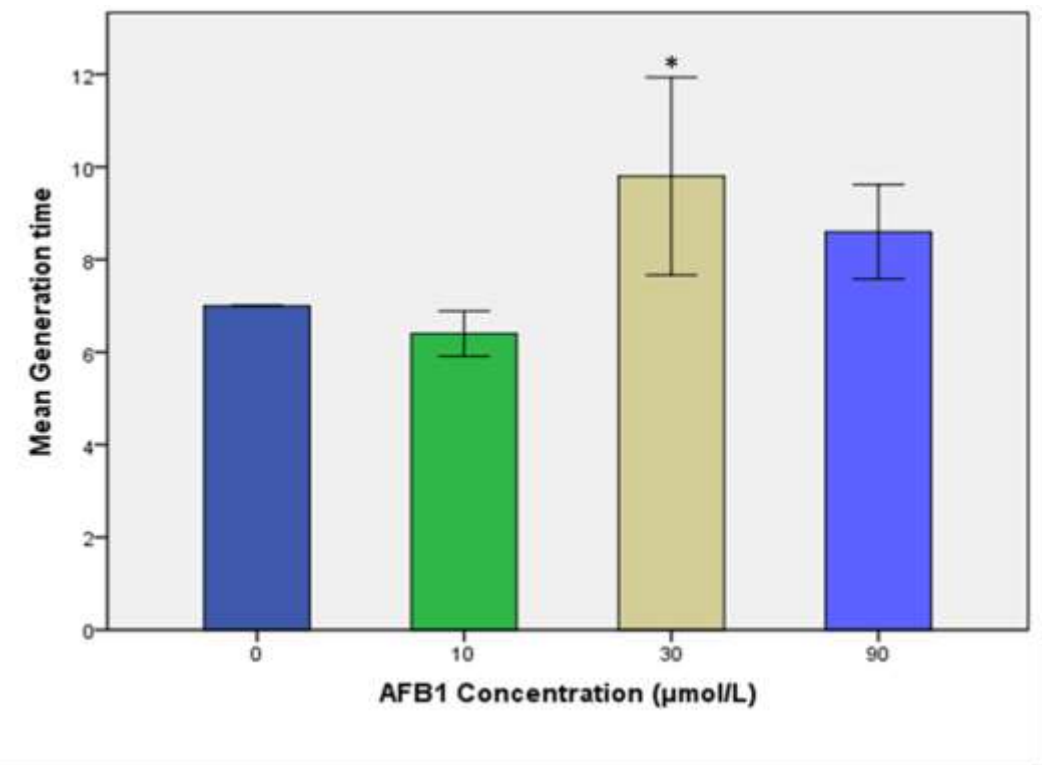

Figure 17: Effect of AFB_1 on generation time in F_2 generation of *C. elegans* (N2 strain). Each bar represents the mean generation time ± SE for 10 adult nematodes per AFB_1 treatment group. *= $p < 0.05$, compared to control.

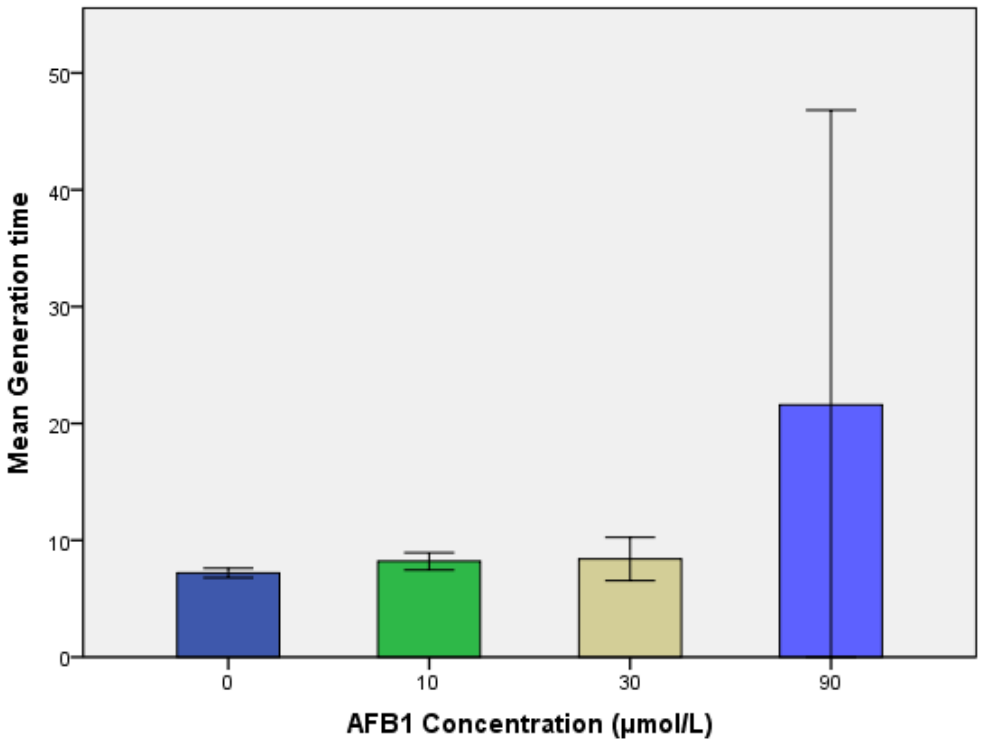

Figure 18: Effect of AFB_1 on generation time in F_2 generation of *C. elegans* (RB strain). Each bar represents the mean generation time ± SE for 10 adult nematodes per AFB_1 treatment group, and no statistical significance ($p > 0.05$) was found.

Table 2: Details of the effect of AFB_1 exposure on growth in the F_0 generation of N2 *C. elegans*. Values represent the mean body length ± SE of 20 nematodes. ***= p<0.0001, compared to control at each time point

Time (hours)	Mean Body length (µm)			
	0 µmol/L AFB1	10 µmol/L AFB1	30 µmol/L AFB1	90 µmol/L AFB1
0	150.15 ± 2.74	150.15 ± 2.74	150.15 ± 2.74	150.15 ± 2.74
24	424.75 ± 4.61	317.45 ± 3.98***	311.05 ± 3.94***	213.40 ± 3.27***
48	811.25 ± 6.37	523.70 ± 5.12***	367.75 ± 4.29***	223.10 ± 3.34***
72	968.65 ± 6.96	728.95 ± 6.04***	536.00 ± 5.18***	307.30 ± 3.92***

Table 3: EC_{50} values and their corresponding 95% confidence intervals for the N2 *C. elegans* F_0 generation Growth experiment

Time (hours)	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
24	15.82	12.49, 20.09
48	12.03	11.05, 13.1
72	16.78	15.34, 18.55

Table 4: Details of the effect of AFB_1 exposure on growth in the F_0 generation of RB *C. elegans*. Values represent the mean body length ± SE of 20 nematodes.
***= p<0.0001, compared to control at each time point

Time (hours)	Mean Body length (µm)			
	0 µmol/L AFB_1	10 µmol/L AFB_1	30 µmol/L AFB_1	90 µmol/L AFB_1
0	99.3 ± 2.23	99.3 ± 2.23	99.3 ± 2.23	99.3 ± 2.23
24	210.5 ± 3.24	147.1 ± 2.71***	131.5 ± 2.56***	103.75 ± 2.28***
48	423.6 ± 4.60	285.75 ± 3.78***	179.55 ± 2.99***	115.45 ± 2.40***
72	724.85 ± 6.02	376.9 ± 4.34***	180 ± 3***	106.95 ± 2.31***

Table 5: EC_{50} values and their corresponding 95% confidence intervals for the RB *C. elegans* F_0 generation Growth experiment.

Time (hours)	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
24	9.34	7.47, 11.66
48	12.31	11.01, 13.76
72	9.4	8.81, 10.04

Table 6: Details of the effect of AFB_1 on brood size in F_0 generation of N2 *C. elegans*. Each value represents the mean brood size ± standard error of two independent experiments
***= p<0.0001, compared to control (0 µmol/L)

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	226 ± 7
10	101 ± 4***
30	18 ± 2***
90	10 ± 1***

Table 7: Details of the effect of AFB_1 on brood size in F_0 generation of RB *C. elegans*. Each value represents the mean brood size ± standard error of two independent experiments
***= $p<0.0001$, compared to control (0 µmol/L)

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	175 ± 5
10	46 ± 3***
30	29 ± 2***
90	4 ± 1***

Table 8: EC_{50} values and their corresponding 95% confidence intervals for F_0 generation brood size experiments.

C. elegans Strain	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
N2	9.94	7.02, 14.09
RB	9.37	3.42, 25.64

Table 9: Details of the effect of AFB_1 on generation time in F_0 generation of N2 *C. elegans*. Each value is the mean of two independent experiments ± standard error
**= $p<0.001$, compared to the control (0 µmol/L)

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	7.2 ± 1.2
10	8 ± 1.27
30	9.4 ± 1.37
90	15 ± 1.73**

Table 10: Details of the effect of AFB_1 on generation time of F_0 generation of RB *C. elegans*. Each value is the mean generation time ± SE for 10 adult nematodes per AFB_1 treatment group. *=p<0.05 and ***=p<0.0001, compared to the control

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	9 ± 9.92
10	11 ± 9.92
30	42.8 ± 9.92*
90	66 ± 9.92***

Table 11: EC_{50} values and their corresponding 95% confidence intervals for the Generation time experiments in F_0 generation.

C. elegans Strain	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
N2	36.7	16.32, 82.52
RB	26.94	25.26, 28.74

Table 12: Details of the effect of AFB_1 on growth in F_1 generation of N2 *C. elegans*. Values represent the mean body length ± SE of 20 nematodes.
*=p<0.05, compared to control at each time point

Time (hours)	Mean Body length (µm)			
	0 µmol/L AFB1	10 µmol/L AFB1	30 µmol/L AFB1	90 µmol/L AFB1
0	211.65 ± 105.17	211.80 ± 105.17	211.15 ± 105.17	182.70 ± 105.17*
24	576.40 ± 105.17	569.30 ± 105.17	564.40 ± 105.17	523.30 ± 105.17*
48	927.55 ± 105.17	860.35 ± 105.17*	837.75 ± 105.17*	784.55 ± 105.17*
72	1032.25 ± 105.17	964.50 ± 105.17*	940.20 ± 105.17*	885.95 ± 105.17*

Table 13: EC_{50} Values and their corresponding confidence intervals for N2 *C. elegans* F_1 generation growth experiment.

Time (hours)	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
24	38.05	5.99, 242
48	14.72	7.52, 28.84
72	14.84	8.47, 26.02

Table 14: Details of the effect of AFB_1 on brood size in F_1 generation of N2 *C. elegans*. *= $p<0.05$, compared to control

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	281 ± 28
10	215 ± 28
30	202 ± 28
90	162 ± 28*

Table 15: Details of the effect of AFB_1 on brood size in F_1 generation of RB *C. elegans*. *= $p<0.05$, compared to control

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	267 ± 31
10	99 ± 34*
30	138 ± 38
90	8 ± 44*

Table 16: EC_{50} values and their corresponding 95% confidence intervals for the F_1 generation brood size experiments.

C. elegans strains	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
N2	12.11	4.19, 34.96
RB	7.27	4.37, 12.09

Table 17: Details of the effect of AFB_1 on generation time in F_1 generation of N2 *C. elegans*. *=$p<0.05$, compared to control.

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	7 ± 2.19
10	14.2 ± 2.19*
30	7.2 ± 2.19
90	9 ± 2.19

Table 18: Details of the effect of AFB_1 on generation time in F_1 generation of RB *C. elegans*. *=$p<0.05$, compared to control.

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	5.6 ± 5.16
10	13.2 ± 5.16
30	17.8 ± 5.16
90	22.6 ± 5.16*

Table 19: EC_{50} values and their corresponding 95% confidence intervals for the generation time experiments in F_1 generation of *C. elegans*.

Generation of nematodes	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
RB	13.49	5.93, 30.73

Table 20: Details of the effect of AFB_1 on growth in F_2 generation of N2 *C. elegans*. Values represent the mean body length ± SE of 20 nematodes.
*=p<0.05, **=p<0.001 and ***=p<0.0001, compared to control at each time point

Time (hours)	Mean Body length (µm)			
	0 µmol/L AFB1	10 µmol/L AFB1	30 µmol/L AFB1	90 µmol/L AFB1
0	112.75 ± 16.88	96.9 ± 16.88	81.2 ± 16.88*	95.45 ± 16.88*
24	226.95 ± 16.88	232.65 ± 16.88	195.05 ± 16.88*	221.5 ± 16.88
48	617.5 ± 16.88	602.5 ± 16.88	537 ± 16.88*	519.6 ± 16.88*
72	948.6 ± 16.88	905.56 ± 16.88*	740.55 ± 16.88***	841.45 ± 16.88**

Table 21: EC_{50} Values and their corresponding confidence intervals for N2 *C. elegans* F_2 generation growth experiment.

Time (hours)	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
24	46.40	10.56, 203.9
48	17.84	12.65, 25.16

Table 22: Details of the effect of AFB_1 on brood size in F_2 generation of N2 *C. elegans*.

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	260 ± 36
10	281 ± 36
30	224 ± 36
90	245 ± 36

Table 23: Details of the effect of AFB_1 on brood size in F_2 generation of RB *C. elegans*. **= p<0.001, ***=p<0.0001, compared to control

AFB_1 Concentration (µmol/L)	Mean Brood size ± SE
0	263 ± 12
10	244 ± 13
30	177 ± 12**
90	7 ± 13***

Table 24: EC_{50} values and their corresponding 95% confidence intervals for the F_2 generation brood size experiments.

Generation of nematodes	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
N2	17.12	0.1349, 2172
RB	35.38	25.38, 49.31

Table 25: Details of the effect of AFB_1 on generation time in F_2 generation of N2 *C. elegans*. *= $p < 0.05$, compared to control.

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	7 ± 0.6
10	6.4 ± 0.6
30	9.8 ± 0.6*
90	8.6 ± 0.6

Table 26: Details of the effect of AFB_1 on generation time in F_2 generation of RB *C. elegans*.

AFB_1 Concentration (µmol/L)	Generation time (h) ± SE
0	7.2 ± 6.33
10	8.2 ± 6.33
30	8.4 ± 6.33
90	21.6 ± 6.33

Table 27: EC_{50} values and their corresponding 95% confidence intervals for the generation time experiments in the F_2 generation of *C. elegans*.

Generation of nematodes	EC_{50} (µmol/L AFB_1)	95% CI (µmol/L AFB_1)
N2	17.35	Very wide
RB	39.19	0.0018, 839247

CHAPTER 5

DISCUSSION

Health effects of AFB_1 exposure in human populations have been studied extensively in epidemiological studies (Lewis *et al*., 2005; Xu *et al*., 2010). Effects of AFs on various parameters has also been investigated using many *in vitro* and animal models (Macé *et al.*, 1997; Qian *et al*., 2014). Most studies testing the toxicological effects of AFB_1 exposure have been focused on its carcinogenic, hepatotoxic and genotoxic effects, especially based on the fact that the effect of AFB_1 was first discovered due to histological evidence of its impact upon organs such as the liver, kidney and other related organs in animals (Blount, 1961) and humans (Shank, 1981). The overarching goal of this study is to add more knowledge to what is presently known concerning how the AFB_1 causes reproductive and developmental toxicity in *C. elegans* and whether there are probable transgenerational effects on F_1 and F_2 generation offspring following AFB_1 exposure in parent (F_0) nematodes. Diseases like stunting of growth and developmental disorders in babies are continually linked to AFB_1 exposure in many developing countries around the world. This is the reason for the conduction of my thesis research to provide more insights about the global problem and to help in developing mitigation strategies to combat the health-related issues as a result of AFB_1 exposure.

L1 stage *C. elegans* were exposed to 0, 10, 30 and 90 µmol/L AFB_1 to investigate the growth inhibitory effects of AFB_1 exposure from early stage nematodes up to adult stage. Our results showed that AFB_1 significantly inhibited the growth of N2 and RB *C. elegans* ($p<0.0001$) in their mean body lengths at each time point of measurement as compared to the control group.

Our results are consistent with previous studies, one of which found growth faltering in animals treated with AFB_1 (Eaton & Groopman, 2013).

To assess the effect of AFB_1 on reproduction in *C. elegans*, L4 stage F_0 nematodes were exposed to 0, 10, 30 and 90 µmol/L AFB_1 to evaluate their effects on the reproductive stage of the nematodes based on their life cycles. The low concentration of AFB_1 (10 µmol/L) that we used in this study has been previously identified to be an environmentally relevant exposure level of AFB_1 (Probst, Njapau, & Cotty, 2007). While the medium and high AFB_1 concentrations (30 and 90 µmol/L respectively) we chose in this study were based on a previous AFB_1 exposure study in *C. elegans* (Leung *et al*., 2010) that showed significant concentration-dependent damage to the DNA after two days exposure to 30 and 100 µmol/L of AFB_1.

In the parental (F_0 generation) generation of our study, we found that AFB_1 exposure caused significant impairment in reproduction of the two strains of *C. elegans* tested. Results from the brood size experiments showed that 10, 30 and 90 µmol/L AFB_1 exposure caused reduction in the mean brood size of N2 *C. elegans* by 47%, 89% and 91%, respectively, as compared to the control group. A similar concentration-dependent reduction was observed in mean brood size of the RB *C. elegans* as well. Results in both strains showed that the most significant impairment in reproduction occurred in the highest concentration (i.e. 90 µmol/L) group. The mean generation time of N2 *C. elegans* exposed to 90 µmol/L AFB_1 was significantly extended with a 2-fold ($p<0.001$) increase as compared to the control group. The mean generation time was more significantly extended in the RB *C. elegans* exposed to 30 and 90 µmol/L AFB_1 with a 4-fold and 6-fold increase ($p<0.0001$), respectively, as compared to the control group. These results showed significant negative effects of AFB_1 exposure on

reproduction in *C. elegans*, which is consistent with a previous study of AFB_1 exposure in poultry (Bryden, Lloyd, & Cumming, 1980) where reduced egg production found in AFB_1-exposed group compared to the control group.

The results of the growth experiment in the F_1 generation showed that the growth inhibitory effects of AFB_1 exposure that was observed in the F_0 generation was transmitted to the next generation. As it was expected since the F_1 generation *C. elegans* were not exposed to the AFB_1 test solutions directly, nematodes across the AFB_1 treatment groups including control group showed improved growth compared to the F_0 generation nematodes. However, a similar concentration-dependent decrease in body length was observed at the different time points of measurement (i.e after 0, 24, 48 and 72 hours) indicating that F_1 generation nematodes may have been exposed to some amount of AFB_1 *in utero*. The result of our study is consistent with a previous study that reported the occurrence of growth defect in neonates as a result of transplacental exposure to AF in gestating pigs (Mocchegiani *et al*., 1998). The gestating pigs were exposed to similar level of AF (800ppb) that have been previously reported to be detected in AF contaminated maize consumed by humans in some countries of Sub-Saharan Africa (Turner *et al*., 2007). Stunting of growth was observed in the offspring of exposed animals indicating that maternal AF exposure during pregnancy can translate to *in utero* toxicity for the next generation.

The mean brood sizes in F_1 *C. elegans* were significantly reduced by 70%, 48% and 97%, respectively, in the 10, 30 and 90 µmol/L AFB_1-treated F_0 nematodes groups as compared to the control group. However, the mean generation time in F_1 *C. elegans* significantly extended with a 4-fold increase only in the highest AFB_1-treated F_0 nematodes group (i.e. 90 µmol/L) ($p<0.05$) as compared to the control group. No statistical significance was found in the 10 or 30

µmol/L AFB_1-treated F_0 groups (p=0.114). Studies investigating the transgenerational effect of AFB_1 are very limited in the literature with an exception of a study reported with ZEN in pigs (Schoevers *et al*., 2012). The study found that ZEN exposure caused a reduction in the ovarian primordial follicle population of F_1 generation female pigs whose parents (i.e. F_0 generation animals) were exposed to ZEN during pregnancy and it was suggested that the effect caused may lead to a premature exhaustion of the follicle pool and risk of reduced litter sizes. Further studies to investigate effects of AFB_1 exposure in the F_1 generation using other established animal models would provide more insights about the present observations found in our study. The fact that similar reproductive and developmental effects that occurred in AFB_1-exposed F_0 nematodes were observed in the F_1 *C. elegans* suggested that AFB_1 *in utero* exposure may have occurred, and caused the effect on the F_1 generation.

The F_2 *C. elegans* were not directly exposed to AFB_1 but may be affected by the exposed germ cells in F_1 generation (Chamorro-GarcÃa *et al*., 2013) and this could be the reason that less reproductive and developmental effects were found generally from the F_2 generation. Our results in F_2 generation suggested a possible transgenerational effects on growth inhibition, which needs to be further investigated.

This study was conducted using two *C. elegans* strains, the wildtype N2 Bristol and the RB864 (*xpa-1*) ok698 strain which is a nucleotide excision repair-deficient strain. The reason to include a mutant strain in our study is the well-known genotoxic effects of AFB_1 which is a fundamental of AFB_1 toxicity, mutagenicity, and carcinogenicity (Meyer *et al*., 2010; Wang & Groopman, 1999). AFB_1- exposure has been shown to cause significant DNA damage in *C. elegans* (Leung *et al*., 2008; Stergiou & Hengartner, 2004). The results from our study which identified more significant inhibitory effect of AFB_1-exposure on the growth in RB *C. elegans*

than in the wildtype N2 strain are consistent with previous study by Leung and colleagues (Leung *et al*., 2010). This observation provides direct evidence for biologically relevant levels of DNA damage as a mechanism of growth inhibition for AFB_1 exposure (Meyer *et al*., 2010) in *C. elegans*.

CHAPTER 6

CONCLUSION

In this thesis research, we observed that exposure of parent *C. elegans* (F_0 generation) to AFB_1 caused reproductive and developmental toxicities in a concentration-dependent manner. The toxic effects were more significant in the highest AFB_1-concentration group tested (i.e. 90 µmol/L) as it is evidenced by the adverse effects in the brood size, generation time and growth results. We found that the toxic effects of AFB_1 observed in the F_0 generation nematodes were transmitted to their F_1 neonates indicating that the F_1 generation nematodes were probably exposed to AFB_1 *in utero* or affected by germ cell exposure which can be inherited. Our results also showed that the growth inhibitive effect identified in the F_0 and F_1 generation appeared in F_2 generation, but the reproduction effects were gradually disappeared in the F_2 generation. This growth inhibition is probably due to the transgenerational effects of DNA damage at the germ cells, as shown by the more sensitive effects in the DNA repair deficient strain (RB). Detailed transgenerational effects of AFB_1 requires further investigation in other model systems.

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APPENDIX

Reagents for Transgenerational experiments

Lactose Broth

3g beef extract

5g bacto-peptone

5g lactose

Dissolve all in **1000ml** of distilled water

Then sterilize by autoclaving

After autoclaving, allow the solution to cool until it is lukewarm

Using a glass Pasteur pipette, add 4 pipette-full of *E. coli* OP50 broth to the solution

Aerate the solution in a water bath set at 37^0C for 24 hours

After 24 hours, the solution turns murky to indicate the presence of bacteria in it

Flame the mouth of the flask containing the solution and cover the flask's mouth with a double-thick aluminum foil

Store in 4^0C refrigerator.

OP50 Broth (*E. coli* stock solution) 100ml

0.25g NaCl

0.5g Bacto-peptone

Combine all in a small Erinmeyer flask and add **50ml** distilled water

Use cotton wool to plug the mouth of the flask with a 1ml sterile pipette inserted

Autoclave the solution

After autoclaving, allow the solution to cool until it is lukewarm and inoculate using sterile technique a loop of frozen E. coli culture into the broth

Aerate the solution in a water bath set at 37^0C for 24 hours

After aerating for 24 hours, remove the flask containing the broth with the solution now appearing murky indicating bacterial growth

Flame the mouth of the flask with a bunsen flame and cover it with a double-thick aluminum foil

Store in 4^0C refrigerator.

Nematode Growth Medium (NGM) Agar

1.77g KCl

2.25g NaCl

12.75g Bacto-Agar

3.76g Bacto-Peptone

Combine all into a 1000ml Fleaker with magnetic stirrer in it and add **750ml** distilled water

Autoclave the preparation for 35 minutes

After autoclaving, allow the preparation to cool enough to touch the Fleaker

Then add to it these solutions in the following order:

1ml cholesterol

1ml 1M $CaCl_2$

1ml 1M $MgSO_4$

Stir the solution well on a stirrer/hot plate machine and using aseptic technique, dispense **30ml** aliquots standard size plates or **10ml** into smaller ones.

Allow to harden at room temperature and store in 4^0C refrigerator

Cholesterol solution

Add **10ml** of undernatured ethanol to **0.1g** cholesterol in a sterile glass bottle

Heat and shake gently to dissolve the mixture on a stirrer/hot plate machine

Store the solution at room temperature.

S-Basal medium

5.85g NaCl

1g K_2HPO_4

6g KH_2PO_4

1ml of **5mg/ml** cholesterol

Dissolve all in **900ml** distilled water and adjust it up to **1000ml** in volume.

Sterilize the whole solution by autoclaving.

S-complete medium

Add **10ml** of sterile 1M Potassium citrate (pH 6) to **977ml** of S-Basal medium.

Add **3ml** of sterile 1M $CaCl_2$

Add **3ml** of sterile 1M $MgSO_4$

Add **10ml** of Trace metals solution.

1M Potassium citrate (pH 6)

Dissolve **3.24g** Potassium citrate in **10ml** distilled water

Autoclave the solution and store at room temperature

1M $CaCl_2$ (50ml)

Dissolve **5.549g** anhydrous $CaCl_2$ in **50ml** of distilled water

Autoclave the solution and store at room temperature

1M $MgSO_4$ (1000ml)

Dissolve **246.48g** $MgSO_4$. $7H_2O$ in **800ml** distilled water

Adjust the volume up to **1000ml** then autoclave and store sterilized solution at room temperature

Trace metals solution

1.86g Disodium EDTA

0.69g $FeSO_4.7H_2O$

0.2g $MnCl_2.4H_2O$

0.29g $ZnSO_4.7H_2O$

0.025g $CuSO_4.5H_2O$

Dissolve all in **1000ml** of distilled water, then autoclave and store in a dark place.

M9 Buffer

5g NaCl

6g Na_2HPO_4

3g KH_2PO_4

Dissolve all in **800ml** of distilled water

Adjust the volume to **1000ml** and autoclave.

When the solution has cooled enough to be touched, add **1ml** of $MgSO_4$

Bleach Solution

2g Sodium Hydroxide pellets

40ml Clorox (5.25% Sodium Hypochlorite)

160ml distilled water

Dissolve and mix the whole solution with a vortex mixer

Store the solution at room temperature

10% Formalin Solution

5ml 37% Formaldehyde

45ml Distilled water

0.45g NaCl

0.6g Na_2HPO_4 (dibasic/anhydrous)

Dissolve and mix all contents in a 50 ml centrifuge tube with a vortex mixer

Store the solution at room temperature