TOXICOLOGICAL EVALUATIONS ON OCHRATOXIN A

IN NEMATODE *CAENORHABDITIS ELEGANS*

by

JOONSU JANG

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Ochratoxin A (OTA) is a widespread mycotoxin produced by several fungal species, including *Aspergillus ochraceus* and *Penicillium verrucosum*. OTA has been reported to cause nephrotoxicity. In this thesis research, we evaluated the toxicological effects of OTA to *C. elegans* based on multiple endpoints, including growth, reproduction, and locomotion, using high-throughput assays. Our results showed that growth and reproduction in *C. elegans* significantly decreased upon the exposure to OTA with concentration-dependent manners. In addition, locomotion in *C. elegans* exposure of OTA displayed a time-dependent decrease in the motility of worms, but, as time went on, a concentration-dependent increase in the motility was indicated as compared to the control. So far, no study has reported the toxic effects of OTA in *C. elegans*. These results suggested that *C. elegans* can serve as a good model organism for evaluation of toxic effects on OTA and mechanistic studies of OTA-induced adverse health effects.

INDEX WORDS: Ochratoxin A; *Caenorhabditis elegans*; High-throughput assay

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DEDICATION

For my beautiful and glorious homeland, Republic of Korea, which I will forever devote my loyalty to.

For the Republic of Korea Ministry of National Defense and the Army Headquarter, supporting my study in the United States

This work is dedicated to my wife, Sejin Jeon, for her love, support, prayers, and guidance, and to my lovely baby, Ayoon Grace Jeon Jang.

To my parents, Man-Ik Jang and In-Suk Oh, who have helped me become who I am today and encourage me to reach for the stars and do what I love.

I would also like to dedicate this to my sisters, Soyeon and Jeong-An Jang.

Finally, I dedicate this to family and friends who have shown their unending support every step of the way.

May all glory be given to my Lord and Savior, Jesus Christ.

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CHAPTER 1

INTRODUCTION

Ochratoxin A (OTA) is the toxic secondary metabolite of several fungal species, including *Aspergillus ochraceus, A. carbonarius* and *Penicillium verrucosum* (Ciegler, 1972; Frisvad & Filtenborg, 1989). Researchers found *Aspergillus ochraceus* in maize and further extracted the toxic agent from the cultures of this mold, which was then named Ochratoxin A (*A. ochraceus*) (Van Der Merwe et al., 1965). OTA were frequently found in plant products, grains, and animal-derived food products, as well as fermented products, such as beer and coffee (Alshannaq & Yu, 2017; Krogh, 1980; Streit et al., 2013; Streit et al., 2012).

The contamination of OTA in food is an ongoing global concern; many international organizations have conducted risk assessments of OTA and established limits on OTA in foodstuffs and calculated tolerable human intakes of OTA. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) of 100 ng/kg of body weight, and European Union (EU) established the Maximum Levels (ML) for OTA in foodstuffs, ranged from 0.5 µg/kg to 10 µg/kg (European Food Safety Authority, 2006; Joint FAO/WHO Expert Committee on Food Additives, 2008; Kuiper-Goodman et al., 2010; The European Commission, 2006).

Many studies have shown that the major target organ of OTA is the kidney, where it produces cytotoxic and renal carcinogenic effects. Also, considerable evidence suggests

that OTA is related to causes of hepatotoxicity, embryotoxicity, teratogenicity, neurotoxicity, and immunotoxicity in both humans and animals (Joint FAO/WHO Expert Committee on Food Additives, 2001, 2008). Most of the toxicity studies on animal models, including mice, rats, dogs, and pigs, have focused on the development of progressive nephropathy; OTA has been shown to be nephrotoxic in all mammalian species tested (Joint FAO/WHO Expert Committee on Food Additives, 2001). In addition, several genotoxicity studies *in vitro* have been conducted to discover OTA's mode of action as a carcinogen. (Joint FAO/WHO Expert Committee on Food Additives, 2008; Kanisawa & Suzuki, 1978).

A strange kind of chronic nephritis has been observed in former Yugoslavia, Bulgaria, and Romania since the 1920s, though only in small towns on the Danube or its tributaries. Due to this narrow distribution range, the disease was named the Balkan endemic nephropathy (BEN) (Castegnaro et al., 1987). The etiology of BEN is unknown, but various reports have suggested that OTA might be involved in the occurrence of BEN. The hypothesis that OTA caused BEN came about since high OTA contamination in foods was recorded in certain endemic regions of the Balkan Peninsula. Moreover, the OTA adducts with DNA were detected in the kidney tumor biopsies of some Bulgarian patients (Petkova-Bocharova & Castegnaro, 1991; Tanchev & Dorossiev, 1991). However, convincing epidemiological data linking exposure rates to the incidence of these diseases are still inadequate, even though various publications describe the occurrence of increased OTA blood levels in the population of endemic villages. Based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans, the International Agency for Research on Cancer (IARC) classified OTA as

possibly carcinogenic to humans (group 2B) (International Agency for Research on Cancer, 1993). Apart from nephrotoxic and carcinogenic effects, OTA can also produce other serious toxic effects, such as growth retardation (NTP, 1989), decreased locomotion (Dortant et al., 2001), and reproductive malfunction (Malir et al., 2013) in mammalian models; however, the mechanism of these adverse effects have not been fully investigated to date.

To evaluate potential toxic effects of OTA, reliable toxicity tests are necessary. Toxicity testing of OTA is usually performed by using conventional *in vitro* and *in vivo* assays using mammalian models like pigs, mice and rats. However, using these animals is time-consuming and expensive with concern of ethical limitation on the use of animals for toxicity tests. Therefore, other *in vivo* or *in vitro* assays were needed for the toxicological field with their strengths in speed- and cost-efficiency, and without concern of animal welfare issues. As an *in vivo* model, *Caenorhabditis elegans* (*C. elegans*) has several advantages that complement *in vitro* assays. *C. elegans* is a tiny and free-living nematode, and first introduced as a model organism by Dr. Sydney Brenner (Brenner, 1974). Since then, the nematode has been extensively used in research in many fields of cell biological, genetic, biomedical, neurobiological, and environmental toxicology studies, due to its many convenient features: an easy and inexpensive maintenance in laboratory conditions with a diet of *Escherichia coli* (*E. coli*), a short life cycle (~ 3days), a large number (300+) of offspring per nematode, relatively short life span, small and transparent body, and well-characterized genome (*C.elegans* Sequencing Consortium, 1998; Yang et al., 2018). Therefore, the use of whole-organism assays, *C. elegans*, is biologically relevant and allows the study of a functional multicellular unit instead of a

single cell (T. Kaletta & M. O. Hengartner, 2006). Furthermore, the *C. elegans* assay also provides organism-level end points, including growth, reproduction, and locomotion (Boyd, Smith, et al., 2010; Williams et al., 2000). Notwithstanding many advantages in *C. elegans* model, few studies have been undertaken for toxicological effects on mycotoxins in *C. elegans*, and moreover there has been no documented study of OTA with a *C. elegans* model.

The working hypothesis of this thesis is that exposure of OTA to *C. elegans* could induce concentration-dependent alterations on growth and reproduction, and concentration- and time-dependent alterations on locomotion. The objective of this thesis is to evaluate how OTA would adversely impact *C. elegans* under different exposure scenarios via multiple endpoints, including body length and optical density for growth, brood size for reproduction, and basic movement for locomotion. The toxic outcomes of OTA were measured using high-throughput assays (the COPAS Biosort and wMicroTracker); high-throughput platform is an increasingly important task in many fields of toxicology (Boyd, Smith, et al., 2010; T. Kaletta & M. O. Hengartner, 2006; Kinser & Pincus, 2017; Tang et al., 2019). In this thesis research, toxic effects of OTA on growth and reproduction were measured using the COPAS Biosort, and the toxic effect on locomotion was measured using the wMicroTracker.

CHAPTER 2

LITERATURE REVIEW

Introduction

Ochratoxin A (OTA) is a widespread mycotoxin produced by several fungal species, including *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum* (Ciegler, 1972; Frisvad & Filtenborg, 1989). The name of OTA originated from its first isolation from a culture of *Aspergillus ochraceus* in 1965 (Van Der Merwe et al., 1965). OTA has been frequently found in plant products, grains, fermentation products, and even animal-derived food products (Alshannaq & Yu, 2017; Krogh, 1980; Streit et al., 2013; Streit et al., 2012). From the data of food contaminant obtained from 2005 to 2011 (Streit et al., 2013), 25% of the world's food supply was contaminated with OTA, and the average positive concentration detected among samples worldwide was 16 µg/kg with a maximum of 1,589 µg/kg.

OTA is absorbed through the gastrointestinal tract, extensively bound to plasma proteins with considerable variations in serum half-lives across species, and slowly eliminated through urine and feces (Ringot et al., 2006; Studer-Rohr et al., 2000). In humans, the extent of protein-binding reaches 99.98%, which results in a long half-life (35 days) for OTA in the body, allowing it to be detected in human blood (Hagelberg et al., 1989). Because of the long serum half-life and the renal elimination of OTA, the blood and urine concentrations of OTA are considered to represent a convenient

biomarker of OTA exposure. Many studies have used serum OTA as a human biomarker of OTA exposure, and OTA in serum was detected to range between 0.1 and 10 ng/mL (European Food Safety Authority, 2006). Urinary OTA, another potential biomarker of OTA exposure, was also found in patients with kidney and urinary disorders with levels ranging from 0.005 to 0.860 ng/mL (Bui-Klimke & Wu, 2015).

Due to high incidence of detectable OTA exposure in blood and urine, indeed, OTA contamination of food is an ongoing global concern; many international organizations have conducted risk assessments of OTA. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) of 100 ng/kg of body weight, and European Union (EU) established the Maximum Levels (ML) for OTA in foodstuffs, ranged from 0.5 µg/kg to 10 µg/kg. (European Food Safety Authority, 2006; Joint FAO/WHO Expert Committee on Food Additives, 2008; Kuiper-Goodman et al., 2010; The European Commission, 2006).

Many studies have shown that the major target organ of OTA is the kidney, where it produces cytotoxic and renal carcinogenic effects, and OTA is also related to causes of hepatotoxicity, embryotoxicity, teratogenicity, neurotoxicity, and immunotoxicity in both human and animals (Joint FAO/WHO Expert Committee on Food Additives, 2008). Most of the toxicity studies on animal models, including mice, rats, dogs, and pigs, have focused on development of progressive nephropathy; OTA has been shown to be nephrotoxic in all mammalian species tested (Joint FAO/WHO Expert Committee on Food Additives, 2001). For the long-term studies of OTA toxicity, renal and liver tumors were observed in treated mice and rats (Kanisawa & Suzuki, 1978). Several genotoxicity studies of animals have been conducted to discover OTA's mode of action as a

carcinogen. Many developmental OTA studies show that it can cross the placenta and that it is embryotoxic and teratogenic in rats and mice. In addition, OTA is immunotoxic and neurotoxic *in vitro* and *in vivo* (Joint FAO/WHO Expert Committee on Food Additives, 2008). Various reports suggested that OTA might be involved in the occurrence of Balkan Endemic Nephropathy (BEN); BEN was a chronic kidney disease and affected the population in some rural regions near the tributaries of the Danube river in Bulgaria, former Yugoslavia, and Romania. The hypothesis that OTA caused BEN was based on the fact that high OTA contamination in foods was recorded in some endemic areas in Bulgaria and Croatia. Moreover, the OTA adducts with DNA were detected in the kidney tumor biopsies of some Bulgarian patients (Castegnaro et al., 1987). However, no study has been published that establish a direct causality of OTA in the etiology of renal tumor, even though many human exposure studies have shown the occurrence in human blood, urine, and breast milk (International Programme on Chemical Safety & World Health Organization, 1990; Petzinger & Ziegler, 2000). Therefore, based on inadequate evidence for carcinogenicity in humans and sufficient evidence for carcinogenicity in experimental animals, the International Agency for Research on Cancer (IARC) classified OTA as a Group 2B (being possibly carcinogenic to humans) (International Agency for Research on Cancer, 1993).

This review summarizes the basic chemistry and biology of OTA, commonly contaminated food stuffs, worldwide regulations, and OTA toxicity, as well as an attractive invertebrate model for toxicity testing, *Caenorhabditis elegans* (*C. elegans*). Although *C. elegans* is recognized as a model organism for mycotoxins, there is no study

conducted with OTA using *C. elegans* models. Therefore, it is necessary to review studies regarding *C. elegans* as a model organism for OTA toxicity study.

Chemistry and biology of ochratoxin A

Ochratoxins are pentaketides within the group of polyketides derived from the dihydrocoumarins family coupled to beta-phenylalanine. The basic molecular structure of ochratoxins is shown in Figure 2.1. Both OTA and ochratoxin B (OTB) have been found as natural food contaminants. OTB is generally less toxic than OTA and does not inhibit protein biosynthesis in hepatoma cells (Dahlmann et al., 1998). OTA, 7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3-R-methyl isocoumarin linked to L-β-phenylalanine, is the first toxin in ochratoxins to be discovered and is the most toxic of the ochratoxins (Chu & Wilson, 1973). OTA has a chlorine atom on C5 of the dihydro-methyl-isocoumarin ring system, whereas OTB does not possess dihydroisocoumarin chlorine atom (Figure 2.1). In addition to the presence of the chlorine atom, a phenolic hydroxyl group increases toxicity (Fun Sun et al., 1972). The molecular formula of OTA is $C_{20}H_{18}ClNO_6$ and the molecular weight is 403.82 Da. The IUPAC developed formula of OTA is (2S)-2-[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl]amino]-3-phenylpropanoic acid. The Chemical Abstracts Service (CAS) registry number of OTA is 303-47-9 and the Chemical Abstracts (CA) index name is N-[(3R)-5-Chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-carbonyl]-L-phenylalanine. OTA is a white crystalline powder, highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate.

The melting points are 90 and 171 ℃, when recrystallized from benzene (containing 1 mol benzene/mol) or xylene, respectively (Ringot et al., 2006).

OTA is slowly absorbed through the gastrointestinal tract; it is distributed to the blood and binds strongly to serum proteins (mainly albumin) and enters enterohepatic recirculation via biliary secretion (Mitchell et al., 2017). Reabsorption of OTA from the intestine as well as the kidneys causes secondary distribution of OTA in the serum and intestinal contents; accumulation occurs in blood, liver, and kidneys. OTA is then slowly eliminated by urinary and fecal excretions (Ringot et al., 2006). These factors can be used to determine the serum half-life of OTA, varying among species such as 39 hours in mice, 72 hours in pigs, 120 hours in rats, 510 hours in monkeys, and 840 hours in humans. All of which were measured after oral administration (Hagelberg et al., 1989; Petzinger & Ziegler, 2000; Studer-Rohr, 1995).

Ochratoxin A-producing fungi and contamination of food commodities with ochratoxin A

Ochratoxin A is a secondary metabolite of many fungal species belonging to the genera *Aspergillus* and *Penicillium*. OTA was first isolated in 1965 from a culture of *Aspergillus ochraceus* Wilh. grown on sterile maize meal; thus, Ochratoxin was named from the fungal species *A. ochraceus* (Van Der Merwe et al., 1965). Three fungi species, *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum*, have been known as major OTA producers; each species has quite different ecology and physiology. It is important to understand the physiology and ecology of these species, in order to determine which species is responsible for OTA formation in a specific food or region.

(Ciegler, 1972; Frisvad & Filtenborg, 1989; Larsen et al., 2001; Ostry et al., 2013; Pitt, 2002; Van Der Merwe et al., 1965). For example, *A. ochraceus* and *P.verrucosum* can produce OTA 8-37℃ and 0-31℃, respectively (Larsen et al., 2001; Pitt, 2002). This feature makes it possible for OTA to be found in a wide variety of agricultural commodities and regions (Lee & Ryu, 2017).

In brief, *A.ochraceus* is capable of growth between 8 and 37 ℃, with the optimum range at 24-31 ℃. *A.ochraceus* has been reported in a wide range of food products but mostly associated with dried and stored foods. Nuts are a major source, including pecans, pistachios, peanuts, hazelnuts, and walnuts. It has been also isolated from cereals and cereal products, as well as cheese, spices, black olives, cassava, and processed meats. Although many studies state *A. ochraceus* as the main producer of OTA, Wilson *et al*. argued that its importance appears to have been overstated (Joint FAO/WHO Expert Committee on Food Additives, 2008; Ostry et al., 2013; Wilson et al., 2002). Secondly, *A. carbonarius* grows better at higher temperatures than *A. ochraceus*, with a maximum of around 40 ℃. Due to high resistance to sunlight and ultra-violet light, *A. carbonarius* has been isolated from grapes and similar fruits that mature in sunlight and at high temperatures (Wilson et al., 2002). Lastly, *P. verrucosum* can be grown under any conditions but especially occurs at low temperatures between 0 and 31 ℃; the main habitat of *P. verrucosum* ranges across Northern and Central Europe and Canada. *P. verrucosum* can be easily found in cereal crops. It also occurs in meat products and in cheese (Ostry et al., 2013; Wilson et al., 2002).

Importantly, OTA is a stable compound and can tolerate high thermal processing (Boudra et al., 1995). Due to the stability and tolerance, OTA is very difficult to destroy

by common food preparation procedures and can be found in cereal products, beer, wine, grape juice, roasted coffee, and instant coffee (Alshannaq & Yu, 2017): the reported average contamination levels are 0.2, 0.02, 0.32, 0.39, 0.62, and 0.76 µg/kg, respectively (European Food Safety Authority, 2006). Apart from that, once ingested as a feed contaminant, OTA is slowly eliminated from animals by urinary and fecal excretions. OTA has been found in animal-derived food products, such as pork meat, offal, milk, and sausages containing pork blood, which originated from animals raised feedstuffs made from contaminated cereals (Duarte et al., 2012; Ostry et al., 2013). For pork (edible offal), the reported average contamination level was 0.17 µg/kg (European Food Safety Authority, 2006)

Food contamination of OTA is a global concern. Streit *et al*. analyzed a large number of samples (17,316 samples) of feed and feed raw materials from all over the world for an eight-year period from 2004 to 2011. They evaluated the extent of mycotoxin contamination. Overall, 25% of the samples tested positive for OTA and the average and maximum detected concentration are 16 µg/kg and 1,589 µg/kg, respectively. Although the level of OTA contamination is low, exceedingly high concentrations can be found all over the world (Streit et al., 2013).

Moreover, it would be of interest to note that recent surveys conducted in the United States found oat-based products are more commonly contaminated with OTA than other cereal-based products (Lee & Ryu, 2017). Nguyen and Rye (Nguyen & Ryu, 2014) collected a total of 144 breakfast cereal and snack samples, including samples mainly from corn, oat, wheat, and rice, for the presence of OTA from different markets and supermarkets in the U.S. during 2012-2013. The analytical results showed that 75

samples (52%) were contaminated with OTA in the range of 0.10 and 7.43 ng/g. Among the OTA contaminated samples, oat-based samples (84%) have the highest OTA frequency and only ten oat-based samples exceeded the maximum limits for OTA (3µg/kg) set by the European Commission Regulation in cereal-based products (The European Commission, 2006). The same research group also conducted the survey over a two-year period (2012-2014), collecting a total of 489 samples. Similarly, 42% of the samples were contaminated with OTA with the highest incidence occurring in oat-based breakfast cereals (70%, 142/203) (Lee & Ryu, 2015). More significantly, Cappozzo *et al*. found that 30% of 155 infant cereals were contaminated with OTA. The oat-based infant cereals had the highest incidence (59%, 30 of 51) and concentrations in the range of 0.6 to 22.1 ng/g (Cappozzo et al., 2017). Considering the EC maximum levels of OTA is 0.5 µg/kg for cereal-based infant foods, high levels of contamination with OTA are an alarming public health concern (Lee & Ryu, 2017; The European Commission, 2006).

Worldwide regulation of ochratoxin A

The Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The JECFA evaluated OTA for the first time at its thirty-seventh meeting, held in Geneva from June 5 to 14, 1990. The Committee established a provisional tolerable weekly intake (PTWI) of 112 ng/kg of body weight (b.w.). The assessment was calculated from the deterioration of renal function in pigs, for which the lowest-observed-effect level (LOEL) was 8 µg/kg b.w. per day. A 500-fold margin of safety was used to derive the PTWI of OTA. The Committee recommended further studies to elucidate the

role of OTA in nephropathy in pigs and humans and the mechanism of tumor formation (Joint FAO/WHO Expert Committee on Food Additives, 1991).

Since a number of reports noted the occurrence of OTA in food commodities in several countries, JECFA re-evaluated OTA at its forty-fourth meeting in 1995. Although multiple toxicological studies of OTA were conducted, including nephrotoxicity, genotoxicity, and investigations on epidemiology, the Committee did not see any need to change the basis on which the previous assessment of the tolerable intake of OTA was made; the PTWI was reconfirmed, rounding it off to 100ng/kg b.w. per week. The Committee reiterated the requests for further studies on OTA (Joint FAO/WHO Expert Committee on Food Additives, 1995).

The JECFA deliberated at its fifty-sixth meeting in 2001 several new studies on OTA that had become available since their last evaluation of OTA: further studies of absorption, distribution, metabolism, and excretion, toxicological studies on genotoxicity, immunotoxicity, neurotoxicity, embryotoxicity, and hepatotoxicity, and the mechanisms of cytotoxicity and nephrotoxicity. While these studies showed various aspects of OTA, the mechanism of tumor formation was unknown, because both genotoxic and non-genotoxic modes of action had been proposed. In addition, although the committee hypothesized that the intake of OTA was associated with nephropathy in humans, causality has not been established. Therefore, the JECFA retained the PTWI of 100 ng/kg b.w. per week, pending the results of studies on the mechanisms of nephrotoxicity and carcinogenicity, and hoped to review the results when they become available (Joint FAO/WHO Expert Committee on Food Additives, 2001).

In 2008, the JECFA re-evaluated OTA during its sixty-eighth meeting, considering all the data available since the last evaluation: toxicity studies, mode of action in the kidney, analytical methods, sampling protocols, methods of prevention and control against food contamination, and dietary exposure assessment regarding OTA. The new data, however, did not indicate any reason to modify the previous PTWI of 100 ng/kg b.w. per week, so the Committee retained the PTWI (Joint FAO/WHO Expert Committee on Food Additives, 2008).

Codex Committee on Food Additives and Contaminations (CCFAC)

At the thirty-eighth session of the CCFAC on March 22-26, 1999, the Committee discussed several topics regarding OTA, including toxicological evaluations, evaluation of exposure, and maximum limits, as well as a number of conclusions and recommendations. Based on all the data available from the session, the CCFAC concluded that a Codex maximum limit for OTA should be established at a level of 5 µg/kg for cereals and cereal products. In addition, since "prevention is better than a cure," the CCFAC would be asked to establish sampling plans and methods of analysis for OTA in cereals; there was no evaluated sampling plan existing for OTA in cereal grains back then and standardization of methods for determination of OTA in cereals and cereal products was ongoing (Codex Alimentarius Commission, 1999).

European Union (EU)

The European Commission's Scientific Committee for Food (SCF) was asked to conduct an evaluation of certain mycotoxins and gave an opinion on aflatoxins, OTA,

and patulin at the thirty-fifth series on September 23, 1994. The SCF agreed that OTA is a potent nephrotoxin agent, a carcinogen, and genotoxic substance. The SCF provisionally concluded that an "acceptable safe level of daily exposure would fall in the range of a few ng/kg b.w." The SCF recommended reconsidering its opinion in light of new information (Scientific Committee for Food, 1996). In 1998, the SCF revised its previous opinion on OTA with respect to results of new toxicological studies; the SCF focused primarily on the mode of action of OTA carcinogenicity, since many reviews on OTA were published and there was general agreement about the OTA toxicity profile. The SCF concluded that recent data from *in vitro* and *in vivo* tests provided evidence of the genotoxic potential of OTA, although further studies were on-going to elucidate the mechanisms involved in OTA carcinogenicity. Therefore, the SCF recommended reducing exposure to OTA as much as possible, ensuring that estimates of tolerable daily intake (TDI) ranged from 1.2 to 14 ng/kg b.w. (Scientific Committee on Food, 1998).

After several evaluations of the toxicity of OTA, the Commission of the European Communities (Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs) promulgated maximum levels (ML) for OTA in various foodstuffs: raw cereal grains (5 µg/kg), all products derived from cereals (3 µg/kg), dried vine fruit (10 µg/kg), roasted coffee beans and ground roasted coffee (5 µg/kg), soluble coffee (10 µg/kg), wine (2 µg/kg), grape juice(2 µg/kg), baby foods(0.5 µg/kg), and dietary foods for special medical purposes (0.5 µg/kg) (The Commission of the European Communities, 2001).

After setting the MLs for OTA, the European Commission requested that the European Food Safety Authority (EFSA) provided a detailed scientific opinion on the

presence of OTA in animal feed. Based on all the toxicological and mechanistic data, the Scientific Panel of the EFSA established a Tolerable Weekly Intake (TWI) of 120 ng/kg b.w. (European Food Safety Authority, 2006).

In 2006, the European Commission re-announced a new regulation, Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, repealing the previous regulation, EC No 466/2001, by adding new restrictions: spices (15-20 µg/kg), licorice root (20 µg/kg), licorice extract (80 µg/kg), and wheat gluten not sold directly to the consumer (8 µg/kg) (The Commission of the European Communities, 2006).

Ochratoxin A regulations in the United States

Currently, the United States has not set regulatory guidelines for OTA in food and feed. Instead, the US Food and Drug Administration (FDA) has supported good agricultural and manufacturing practices and required the implementation of food safety plans in food industry undertakings to keep the levels of OTA to the lowest level feasible (Park & Troxell, 2002). Mitchell et al. (Mitchell et al., 2017) conducted the first human exposure and risk assessment for dietary OTA exposure in the US population to determine which among the different age and consumption groups whose OTA exposure did not exceed the JECFA PTWI (100 ng/kg b.w. per week or about 14.3 ng/kg b.w. per day) or the Negligible Cancer Risk Intake (NCRI, 4 ng/kg b.w. per day) utilized in the proposed Health Canada MLs. The average OTA exposure across the US population are much lower than the PTWI and NCRI: for ≤12 month, >12 month-5 year, >5−18 year and >18 year old subpopulations, the overall mean exposures were 0.37, 0.39, 0.14, and

0.12 ng/kg b.w. per day, respectively. Therefore, the authors concluded that the US population had negligible risk from OTA exposure, because the associated risks from current food consumptions did not exceed levels of concern.

Toxic effects of ochratoxin A in animals

OTA contributed to the endemic porcine nephropathy in all areas of Denmark during 1970s and 1980s (Krogh, 1976, 1977; Krogh et al., 1973). Following the discovery of animal nephropathies, many studies were conducted to show OTA renal toxicity. After conducting acute toxicity tests, the LD_{50} values were found in various animal species. The order of LD_{50} sensitivity by oral administration was dog, pig, chicken, rat, and mouse; and the values are 0.2, 1, 3.3, 20-30, and 46-58 mg/kg b.w., respectively (Joint FAO/WHO Expert Committee on Food Additives, 2001). Earlier studies focused on the implication of OTA in the renal toxicity, so the nephrotoxicity of OTA was well documented (Elling et al., 1985; Krogh, 1992; Stoev et al., 2002; Stoev et al., 2001). EFSA and JECFA extensively reviewed the toxicity of OTA and concluded that the kidney was determined to be the major target organ for the adverse effects of OTA (European Food Safety Authority, 2006; Joint FAO/WHO Expert Committee on Food Additives, 2008).

OTA has induced renal toxicity in all mammalian species tested. Short-term toxicity studies in mice (Bendele et al., 1985), rats (Boorman et al., 1992), and pigs (Elling et al., 1985) showed that OTA was associated with the development of progressive nephropathy with dose-dependent and time-dependent manners. In porcine studies, Stoev *et al*. reported that mild nephropathy was induced in all experimental pigs

(3 female and 3 male pigs) by a diet containing OTA at 800 ppb for a year. The renal lesions were similar to those described for classical porcine nephropathy formerly encountered in Denmark. They found two types of histological changes: degenerative affecting epithelial cells in some proximal tubules of pigs after 6 months, and proliferative changes in the interstitium of some renal cortical regions after one-year OTA exposure. There were, however, no histopathological changes in kidneys of any of the control pigs. This study also showed a direct relationship between OTA concentrations in feed and OTA concentrations in serum after exposure to contaminated feed in pigs. These results strongly suggested that OTA was associated with the etiology of porcine nephropathy (Stoev et al., 2002). In rodent models, the United States National Toxicology Program (NTP) conducted studies of OTA toxicity and revealed that OTA could induce renal tumors in rodents at high dosages. In these studies, groups of F344/N rats of each sex were given OTA in corn oil by gavage for up to 2 years. Compound-related kidney lesions in rats were found from 16 days and 13 weeks studies, such as renal tubular cell degenerative and regenerative changes (nephropathy), adrenal gland hemorrhage, etc. Administration of OTA for 9 months and 15 months was associated with increased incidences of renal tubular cell neoplasms in male rats and hyperplasia, degeneration, and karyomegaly of renal tubular epithelial cells in both male and female rats. In other words, renal carcinomas were found in a lot of male rats with a diet containing 70 and 210 µg/kg b.w. OTA; no carcinoma was found in male rats at the control(0 µg/kg b.w. per day) and lowest doses (21 µg/kg b.w. per day). In female rats, however, renal carcinomas were less common at all doses (0, 21, 70, 210 µg/kg b.w. per day) (NTP, 1989). While NTP has discovered sex differences in susceptibility to OTA-

induced nephrotoxicity, Dortant *et al*. investigated age-related differences in nephrotoxicity of OTA. The research group conducted a study in young adult (aged 12 weeks) and old (aged 27-30 months) female SPF Wag rats, treated by gavage with OTA doses of 0, 0.07, 0.34 or 1.68 mg/kg b.w. for 4 weeks. The research group found that OTA induced primarily nephropathy for both young adult and old aged groups, but old female rats are more sensitive to induction of tubular karyomegaly and vacuolation / necrosis than young adult female rats. In summary, old female rats are more sensitive to OTA than young adult female rats, although the profiles of OTA toxicity for both age groups are similar (Dortant et al., 2001).

While the abundance of adverse effects shown in animal studies show us that OTA is nephrotoxic, we still do not have adequate data to conclude the mode of carcinogenic action by OTA and the genotoxicity of OTA is still controversial. OTA was shown negative in conventional tests at the levels of gene and chromosome. OTA did not induce reverse mutations in *Salmonella typhimurium* strain TA102 (Würgler et al., 1991). Inhibition of growth did not occur in various strains of *Bacillus subtilis* after OTA treatment (Ueno & Kubota, 1976). No mutagenic effect occurred in *Saccharomyces cerevisiae* strain D-3 under OTA treatment conditions (Kuczuk et al., 1978). OTA did not induce any mutation in FM3A cells, a C3H mouse mammary carcinoma cell line (Umeda et al., 1977). However, Faucet *et al*. conducted a study of rats given a chronic exposure to OTA and of pigs, a sub-chronic exposure, which showed evidence of DNA-adducts formation (Faucet et al., 2004). In addition, DNA adducts formation, isolated from liver, kidney and spleen in mice, has been reported by using ^{32}P-postlabeling after oral treatment with OTA at 0.6, 1.2 and 2.5 mg/kg body weight (Pfohl-Leszkowicz et al.,

1991). A significant increase in OTA-induced mutation frequency was reported in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay in V79 hamster cells as well as in the thymidine kinase (TK) assay in L5178 mouse lymphoma cells, via a mechanism that was independent of biotransformation (Palma et al., 2007). In recent studies, DNA single-strand breaks were reported as assessed by Comet assay in a dose-dependent manner in the liver, kidney and spleen of F344 rat treated with OTA. The studies also found that the extent of DNA damage in liver and kidney was further enhanced in the presence of formamidopyrimidine-DNA glycosylase, which converted oxidative DNA damage into strand breaks, suggesting the presence of oxidative DNA damage (Mally, Pepe, et al., 2005; Mally, Völkel, et al., 2005). As a result, the data on genotoxicity of OTA remain controversial, despite the various reports on DNA adduct formation, gene mutations, and oxidative DNA damages. However, the studies of OTA genotoxicity provide suggestive evidence for the role of oxidative stress in nephropathologies associated with OTA (European Food Safety Authority, 2006).

Furthermore, many studies found that OTA was relevant in causing neurotoxicity, immunotoxicity, embryotoxicity, and teratogenicity in animals. OTA is neurotoxic *in vitro* and *in vivo*. In *in vitro* studies, OTA decreased in viability of both proliferating and differentiating cells with dose- and time-dependent manners in cultured proliferating neural stem/progenitor cells (NSCs) prepared from hippocampus of adult mouse brains. The authors, Sava *et al*., suggested that exposure to OTA may contribute to impaired hippocampal neurogenesis *in vivo*, resulting in depression and memory deficits (Sava et al., 2007). Similarly, in rat brain cells, OTA treatments increased the expression of genes involved in the brain inflammatory system and reduced the expression of glial fibrillary

acidic protein, a constituent of the intermediate filaments in astrocytes (Zurich et al., 2005). In mice, administration of OTA, given intraperitoneally in doses of 0–6 mg/kg b.w., resulted in a depletion of striatal dopamine content with a dose-dependent manner. In addition, OTA treatment increased oxidative stress, oxidative DNA damage, and a transient inhibition of oxidative DNA repair in different brain regions of these mice (cerebellum, cortex, hippocampus, midbrain, caudate/putamen, and pons/medulla) (Sava et al., 2006). The research group of Dortant *et al*. conducted neurotoxicity tests with groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats given OTA by gavage at doses of 0, 0.07, 0.34 or 1.68 mg/kg b.w. per day for 4 weeks. The research group noted that vacuolation of the white brain matter (cerebellar medulla and ventral parts of the brain stem) was largely increased at all OTA dose levels in young rats at 0.34 and 1.68 mg/kg b.w. per day and in old rats at 0.07 and 0.34 mg/kg b.w. per day (Dortant et al., 2001). Thus, OTA has been shown to be highly toxic for the nervous cells and able to reach the neural tissue.

Under certain conditions, OTA shows a great immunosuppressor effect, which is observed at low or high doses. Dortant *et al*. found that OTA induced a dose-related reduction in splenic T-cell fraction in the young rats without significant changes in T-helper and T-suppressor subpopulations. In this study, OTA was given to groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats by gavage at doses of 0, 0.07, 0.34 or 1.68 mg/kg b.w. per day for 4 weeks. Serum immunoglobulin G (IgG) levels significantly decreased at 0.34 mg/kg OTA (young and old rats) and 1.68 mg/kg OTA (young rats); on the other hand, serum immunoglobulin M (IgM) levels relatively increased at 0.07 mg/kg in young rats only. As a result, Dortant *et al*. suggested

that the immune system was a possible OTA toxicity target, but probably not the most sensitive one (Dortant et al., 2001). In another rat study, Alvarez *et al*. investigated immunotoxicity of OTA in Wistar male rats (aged 12 weeks), treated by gavage with 50, 150 or 450 mg OTA/kg b.w. for 28 days. The response of splenocytes to sheep red blood cells lowered in a dose-dependent manner; however, the differences were statistically not significant. Also, OTA treatment affected the natural killer cell activity and decreased cytotoxic T lymphocyte activity in the animals. The bacteriolytic capability of macrophages was significantly decreased after OTA treatment. The study concluded that OTA could affect immune function in rodent models. (Álvarez et al., 2004). Following the results of these studies, OTA was clearly seen as an important immunosuppressor agent (el Khoury & Atoui, 2010).

OTA is a potent teratogen in rats and rabbits. To determine the teratogenic effects, groups of pregnant Wistar rats were given OTA orally dissolved in corn oil at doses of 0.125, 0.25, 0.50, and 0.75 mg/kg during 6-15 days of gestation. OTA induced a dose-dependent increase in the fetal gross, skeletal, and visceral anomalies in fetus, as compared with those of the control. Major gross anomalies observed included exencephaly (incomplete closure of skull), micrognathia, micromyelia, scoliosis (bent spinal cord), and small hind portion. The major skeletal defects involved skull bones, ribs, sternebrae (malposition or missing), and vertebrae (failure or incomplete ossification) (Wangikar, Dwivedi, & Sinha, 2004). In addition, histopathological examination in the tissue sections showed dose-dependent increase in fetal liver, kidneys, brain, heart, and eyes in rats with a dose-dependent manner (Wangikar, Dwivedi, Sharma, et al., 2004). In a rabbit study, groups of New Zealand White rabbits (five per

group) were given OTA by oral gavage in corn oil at doses of 0, 0.025, 0.050, and 0.10 mg/kg b.w. per day during days 6–18 of gestation. The research group found a significant depletion in fetal weight, number of live fetus. In addition, certain anomalies were observed following the treatment of OTA such as skeletal and visceral anomalies, e.g. knuckling of fetlock, rudimentary tail or agenesis of tail, wavy ribs, hydrocephalus, microphthalmia and agenesis of kidney. Certain histopathological anomalies were observed in liver, kidney, brain, and eyes, following the treatment of OTA (Wangikar et al., 2005). To sum up, these studies noted that OTA could cross the placenta and accumulate in fetal tissues causing various morphological anomalies (el Khoury & Atoui, 2010).

Toxic effects of ochratoxin A in human populations

Although the abundant adverse effects of OTA have been shown in animal studies, no documented epidemiological evidence of OTA exposure causing adverse effects in humans have been reported (Pfohl-Leszkowicz & Manderville, 2007). However, many scientists have investigated the possibility of OTA-related toxicity in humans: studies conducted in several countries, including Balkan Endemic Nephropathy regions, Egypt, and Tunisia founded that higher serum or plasma OTA levels in patients might be associated with kidney and urinary disorders compared to healthy control groups, although the associations may not be causal (Bui-Klimke & Wu, 2015)

Balkan endemic nephropathy (BEN) was a chronic kidney disease and affected the population in certain rural regions near the tributaries of the Danube river in Bulgaria, former Yugoslavia, and Romania. This disease was associated with cancer of the upper

urinary track (UUT) (Miletić-Medved et al., 2005). Krogh (Krogh, 1972) found that OTA might be involved in the etiology of BEN, because the histopathology of BEN was similar to OTA-induced porcine nephropathy. In addition, Castegnaro *et al*. (Castegnaro et al., 2006) noted that OTA was found frequently at high average concentrations in blood samples from people living in Bulgaria: the OTA concentrations in blood were from 260 to 8360 µg/L, which was much higher than the previous data (10 µg/L) in the same area (Petkova-Bocharova et al., 1988). Petkova-Bocharova and Castegnaro (Petkova-Bocharova & Castegnaro, 1991) conducted an experiment to determine the occurrence of OTA in blood from people living in the endemic area who were either affected or unaffected by BEN and urinary tract tumors (UTT) and in blood from people living in control regions where these diseases did not occur. The research team indicated elevated levels of OTA in the blood from the patients with urinary system tumor and endemic nephropathy living in BEN-endemic villages than in the blood from unaffected people from endemic villages and control areas (nonendemic villages): the difference between the proportion of ochratoxin A-positive samples from the patients group (26.3%) and those from unaffected people from endemic villages (11.6%) and unaffected people from control areas (7.7%) was statistically significant ($P < 0.02$ and $P < 0.001$, respectively). Hence, many researchers supposed that exposure to OTA was associated with BEN and/or UTT, although no data has been published that establish a direct causality of OTA exposure in the etiology of BEN and UTT.

Similarly, in Egypt, Wafa *et al*. conducted a study to delineate the size of the problem of ochratoxicosis and its relation to genesis of lesions mounting to end stage renal disease (ESRD) or urothelial tumors. The research team found that high ochratoxin

serum levels were found in patients with ESRD and nephrotic syndrome. Also, positive serum, urine and tissue biopsy specimens for ochratoxin levels were found in the group with urothelial tumor. They concluded that OTA could be correlated to the genesis of renal disease leading to ESRD or causing urothelial cancer (Wafa et al., 1998). In areas in North Africa, like Tunisia where disease symptoms with high incidence of chronic interstitial nephropathies of unknown etiology were prevalent, OTA was suspected to play a major role, but contradictory studies on the degree of human exposure failed in evidencing the role of OTA. In one study, the determination of OTA in human blood was conducted in a Tunisian population. The study found that elevations in serum OTA were greatest in those diagnosed with chronic interstitial nephropathy (CIN) at mean values of 25-59 ng/ml compared to in the general population at mean values of 0.7-7.8 ng/ml and in those with other types of kidney disease at mean values of 6-18 ng/ml (Maaroufi et al., 1995). Another Tunisian study attempted to confirm the relationship between OTA blood levels and the development of renal pathology, so the research team measured serum OTA levels in several groups of patients having different renal diseases. 49% of the healthy group showed OTA concentrations ranging from 1.7 to 8.5 ng/ml, with a mean value of 3.3 ± 1.5 ng/ml. However, people with CIN of unknown etiology showed the highest incidence in OTA concentrations (76%), ranging from 1.8 to 65 ng/ml with a mean value of 18±7 ng/ml. This study hypothesized that OTA was involved in the outcome of particular human nephropathy (Zaied et al., 2011).

In conclusion, while these studies show us the possible role of OTA in human nephropathies and urinary tract tumors, no adequate human studies of the relationship

between exposure to OTA and human cancer have been reported (el Khoury & Atoui, 2010).

Caenorhabditis elegans as a novel animal model for mycotoxins

Caenorhabditis elegans

C. elegans is a tiny and free-living nematode (roundworm) about 1mm in length and most easily isolated from rotting vegetables, which is a sufficient supply of their bacterial food source (Barrière & Félix, 2014).*C. elegans* was first introduced as a model organism by Dr. Sydney Brenner (Brenner, 1974). Since then, it has been extensively used in a variety of biological studies, due to its many convenient features.

First, *C. elegans* is easy and inexpensive to maintain in laboratory conditions. *C. elegans* can be cultured on nematode growth medium (NGM) plates with *Escherichia coli* (*E. coli*) as a food source. The nematodes can also be cultured in a liquid medium, such as M9 buffer or K-medium with *E. coli* (Williams & Dusenbery, 1988). In addition, *C. elegans* can be frozen at −80℃ for over ten years, but survival is not as great as for worms stored in liquid nitrogen at −196℃ (Brenner, 1974): starved L1-L2 stage nematodes survive in freezing condition well, but well-fed animals, adults, eggs, and dauers do not (Stiernagle, 2006). *C. elegans* has a short life cycle of about three days at 25℃ from egg through the four stages of larva (L1 – L4) to mature adults (Figure 2.2), the normal life span of approximately 2-3 weeks, and a generation time of approximately 4 days under laboratory conditions.

Second, the reproduction of the nematodes is robust; the adult hermaphrodite can produce about 300 eggs by self-fertilization and over 1,000 eggs, when inseminated by a

male. *C. elegans* has two sexes: hermaphrodites and males. The most common form of nematodes is the hermaphrodite, but males are at less than 0.2% of the total population on average (Corsi et al., 2015). The basic anatomy of *C. elegans* includes a mouth, pharynx, intestine, gonad, and collagenous cuticle. Males have a single-lobed gonad, vas deferens, and a tail specialized for mating. Hermaphrodites have two ovaries, oviducts, spermatheca, and a single uterus. The hermaphrodites produce sperms first in the L4 stage and then switch over to producing oocytes (Corsi et al., 2015).

Third, the genetics of *C. elegans* is well developed and is the first animal whose genome is completely sequenced (*C.elegans* Sequencing Consortium, 1998). It has been showed that *C. elegans* has about 20,000 genes, about 38% of the *C. elegans* protein-coding genes have recognizable homologs in humans (Shaye & Greenwald, 2011), 40% of human disease genes have clear orthologs in the *C. elegans* genome (Culetto & Sattelle, 2000), and 60-80% of human genes are ortholog in the *C. elegans* genome (Titus Kaletta & Michael O. Hengartner, 2006). *C. elegans* has five pairs of autosomes and one pair of sex chromosomes. Hermaphrodite *C. elegans* have a pair of sex chromosomes (XX); the males have a single sex chromosome (X0). RNA interference (RNAi) has been extensively used on *C. elegans*; the function of specific genes can be disrupted by RNAi (Fire et al., 1998). Using gene silencing technique by RNAi, scientists were able to knock down more than 20,000 genes in the worm, establishing a functional role for 9% of the genome (Ahringer, 2006). In addition, the development of *C. elegans* is well documented. It is known that the adult hermaphrodite *C. elegans* has 959 somatic nuclei, 302 of which are neurons and 95 are body wall muscle cells, whereas the adult male has 1031 somatic nuclei and 381 of these are neurons (White et al., 1986). Its complete cell

lineage has been established and can be traced at each developmental stage (Kipreos, 2005).

Toxicity studies on environmental contaminants with C. elegans

C. elegans has been widely used to evaluate the toxicity and toxicological mechanisms of environmental contaminants, including heavy metals and manufactured nanoparticles. Generally, these studies elucidated the toxicity with various toxic endpoints in *C. elegans*, such as lethality, growth, reproduction, and behavior (movement or feeding), as well as transgenic expression in a transgenic strain of *C. elegans.*

Williams and Dusenbery (Williams & Dusenbery, 1988) designed a toxicity screening test to rank the toxicity of eight metallic salts, $HgCl_2$, $BeSO_4 \cdot 4H_2O$, $Al(NO_3)_3 \cdot 9H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $Pb(NO_3)_2$, $CdCl_2$, and $Sr(NO_3)_2$, based on *C. elegans* lethality, and the LC_{50}s of the eight metallic salts compared with published rodent oral LD_{50} values for the same metallic salts. They found that the ranking of the LC_{50} values in *C. elegans* was statistically consistent with the order of rodent oral LD_{50}s. This study suggested that toxicity tests using LC_{50} raking in *C. elegans* might play an important role of expecting LD_{50} ranking in rats or mice, because the *C. elegans* acute lethality tests costed about 10% of the cost for the rodent tests. In another study, multiple endpoints, including movement, feeding, growth, and reproduction, were used to characterize the sensitivity for heavy metal (Cd, Cu, and Pb) toxicity testing with *C. elegans* (Anderson et al., 2001). They found that the behavior endpoints of feeding and movement in *C. elegans* were more sensitive to neurotoxicants than other sublethal endpoints, such as growth and reproduction. Apart from that, Ma *et al*. (Ma, Glenn, et al.,

2009) conducted a toxicity test for heavy metals (Cd, Hg, Cu, Zn, Ni, and Pb) with a transgenic strain of *C. elegans* (*mtl-2::GFP* transgenic *C. elegans*), which used the *C. elegans* MT-2 (*mtl-2*) promoter to control the transcription of green fluorescence protein (GFP) reporter. Toxic effects on the exposure of these heavy metals to the transgenic strain of *C. elegans* were measured by quantifying GFP expression after 24-hour exposure. They found that Cd, Hg, Cu, and Zn induced GFP with a time- and concentration- dependent manners, whereas Ni and Pd did not. Likewise, the nematodes exposed to Cd, Hg, Cu, and Zn induced upregulated *mtl-2* transcription compared to the control, but Ni and Pb were downregulated. The results suggested that using *mtl-2::GFP* transgenic strain of *C. elegans* provided easier, faster, and cheaper method for measuring metallothionein induction in response to heavy metal exposure, compared to existing assay methods.

In addition, *C. elegans* has been investigated in toxicity studies for evaluating the potential toxicological effects of manufactured nanoparticles. Ma *et al*. (Ma, Bertsch, et al., 2009) investigated toxicity of manufactured zinc oxide nanoparticles (ZnO-NPs; 1.5 nm) compared to aqueous zinc chloride ($ZnCl_2$) in *C. elegans*, using multiple endpoints of lethality, behavior, reproduction, and transgene expression in a *mtl-2::GFP* transgenic strain of *C. elegans*. The results showed that no significant difference was found in lethality, behavior, and reproduction in *C. elegans* between ZnO-NPs and $ZnCl_2$. Also, at a given concentration, ZnO-NPs showed no significant difference from $ZnCl_2$ regarding increases in GFP expression in the transgenic strain of *C. elegan,* suggesting similar intracellular biotransformation between ZnO-NPs and $ZnCl_2$ to *C. elegans*. Given these

results, this study demonstrated that both ZnO-NPs and $ZnCl_2$ had similar toxic effects on *C. elegans*

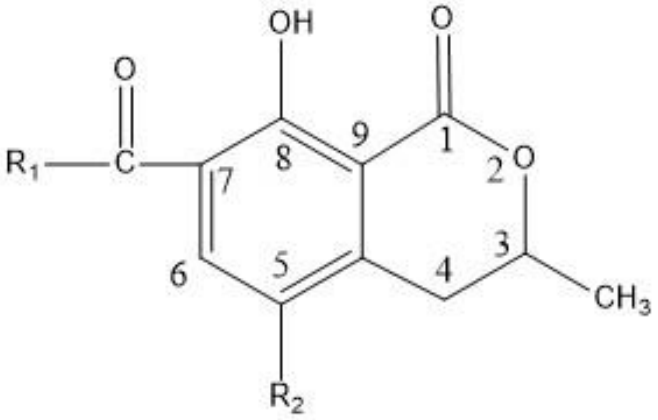
Toxicity studies on mycotoxins with C. elegans

Notwithstanding these unique features in *C. elegans* model, there have been a few studies investigating the toxicological effects on mycotoxins in *C. elegans.* Yang *et al*. (Yang et al., 2015) evaluated the multiple toxic endpoints of common mycotoxins, Aflatoxins B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1), T-2 toxin (T-2), and zearalenone (ZEA), in *C. elegans* model, including lethality, toxic effects on growth and reproduction, as well as influence on lifespan. This study concluded that *C. elegans* model can predict toxic effects of mycotoxins and can assess developmental and reproductive toxic effects of mycotoxins. The same research group (Yang et al., 2018) investigated the possible molecular mechanisms of ZEA-induced reproductive and developmental toxic effects in *C. elegans*. They found that ZEA induced many abnormal vulva morphologies in *C. elegans*. This study concluded that disruption of collagen biosynthetic pathway could be an evidence of ZEA-induced reproductive and developmental toxic effects in *C. elegans*. In another study, Zhou *et al*. (Zhou et al., 2018) used *C. elegans* to evaluate the trans-/multi-generational toxicities of DON through three endpoints: growth, brood size, and feeding ability. They found that DON exposure induced significant trans-/multi-generational toxic effects on *C. elegans*. This result suggested that this study may serve as an important contribution to *C. elegans* model on investigating molecular mechanisms of long-term adverse effects of DON. Similarly, Feng *et al*. (Feng et al., 2016) examined AFB1, which is a potent genotoxin mutagen that

can cause DNA damage, to investigate the link between DNA damage and abnormal development and reproduction, using *C. elegans* as a study model. In this study, several tests, including the DNA damage, germline apoptosis, growth, and reproductive toxicity following exposure to AFB1, were conducted in *C. elegans*. AFB1 induced DNA damage and germline apoptosis, and adversely affected growth and reproduction in a concentration-dependent manner. These results implied that AFB1-induced DNA damage can be associated with growth inhibition and germline apoptosis and result in inhibition of reproduction.

In conclusion, although *C. elegans* has been shown as a model organism for investigating mycotoxin toxicity in various studies, there has been no documented study of OTA with a *C. elegans* model. In addition, mycotoxin studies with *C. elegans* are critical to how we research OTA toxicity under several endpoints, which are used to determine the toxicity of mycotoxins in the studies. Therefore, the *C. elegans* model can be used to study OTA-induced adverse health effects.

Figures



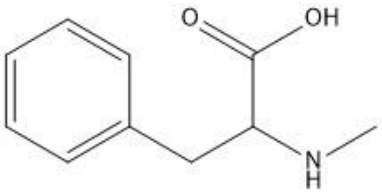
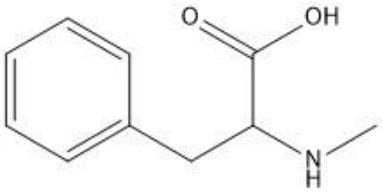
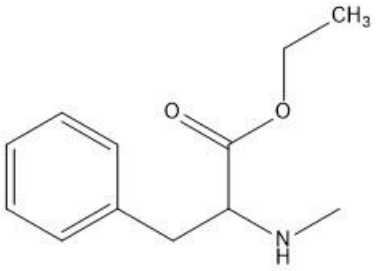
	R_1	R_2
OTA		— Cl
OTB		— H
OTC		— Cl
OTα	— OH	— Cl
OTβ	— OH	— H

Figure 2.1 Molecular structures of ochratoxins

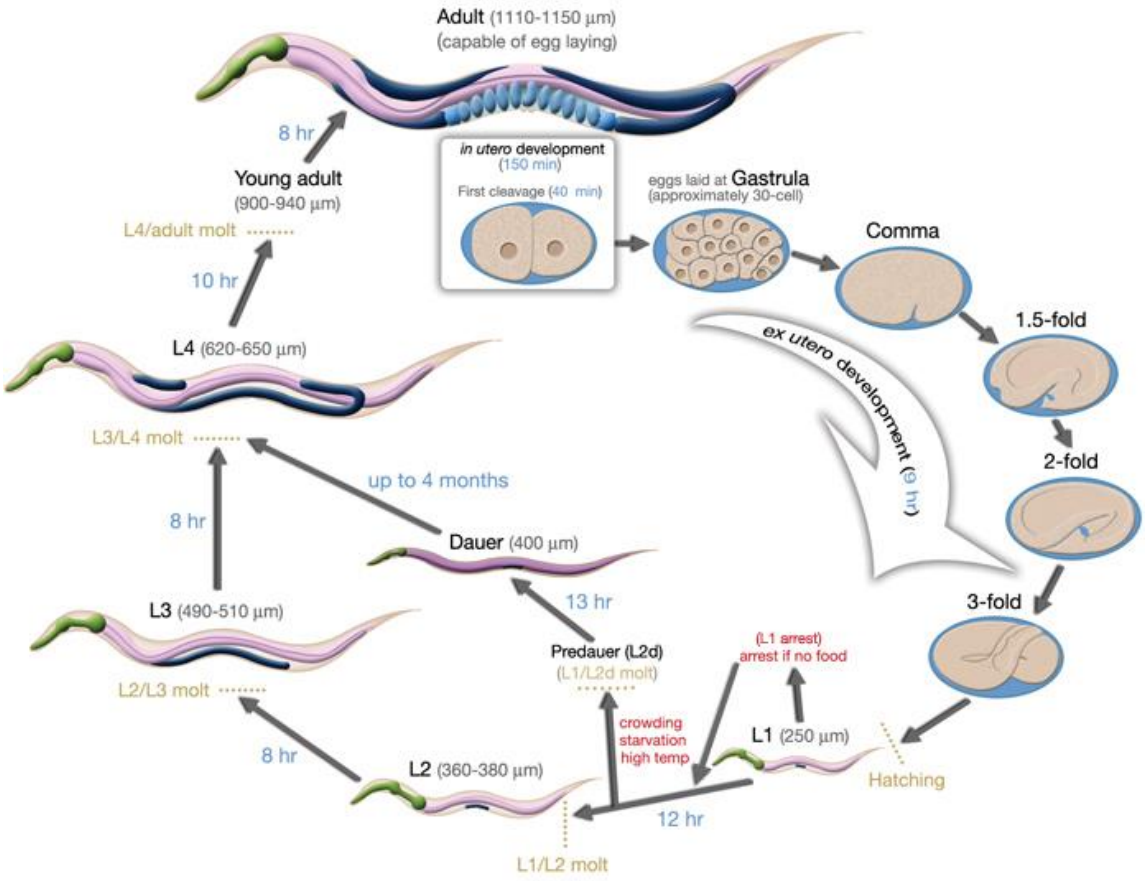


Figure 2.2 The life cycle of *C. elegans*. (Adapted from

https://www.wormatlas.org/hermaphrodite/introduction/mainframe.htm)

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CHAPTER 3

MATERIALS AND METHODS

Chemicals

OTA of ≥98% purity (HPLC grade) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). OTA was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) to prepare a stock solution (12.5 mg/ml). All other chemicals were purchased commercially at the highest degree of purity available.

C. elegans strains and culture conditions

Wild-type Bristol (N2) strain of *C. elegans* was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). All nematodes in the experiments were hermaphrodites. Populations of *C. elegans* were maintained on a K-agar plate (2.36 g KCl, 3 g NaCl, 2.5 g Bacto-peptone, 17 g Bacto-agar, 1 mL cholesterol (5 mg/mL), 1 mL 1 M CaCl2, 1 mL 1 M MgSO4 in 1 L dH2O) seeded with *Escherichia coli* (*E. coli*) strain OP50 as food source, incubated at 20°C (Brenner, 1974; Williams & Dusenbery, 1988), and transferred to a new plate weekly.

Preparation of a large synchronized population of *C. elegans* was recommended for use in high throughput assays in order to reduce variation in results due to developmental differences. Worms could be synchronized by using a bleaching solution (1 g NaOH, 20 mL 5.25% NaOCl, 80 mL dH2O). The bleaching method was based on

the fact that worms were sensitive to bleaching solution, while embryos were protected from the bleaching solution by eggshells. The method was previously described in detail by Boyd *et al* (Boyd et al., 2012). Briefly, some of the maintenance plates were incubated for three days to obtain *C. elegans* eggs, yielding age-synchronized adult nematodes. Adult nematodes and eggs were rinsed from K-agar plates with K-medium (2.36 g KCl, 3 g NaCl, in 1 L dH2O) and transferred to 15-mL centrifuge tubes. The adult nematodes and eggs were centrifuged at 3000 rpm for 7 min in the 15-ml centrifuge tube to make a pellet. The supernatant, which might contain bacteria, was carefully discarded. Then approximately 10mL of bleaching solution was added to each 1.5 mL leftover into the tube to isolate the eggs from nematodes and the mixture was gently agitated for 4 min. The mixture was quickly centrifuged at 3000 rpm for 7min and supernatants were discarded. The pellets were rinsed with K-medium and centrifuged for three times with K-medium, removing the supernatant and leaving the white pellet in the bottom of the tube. After three iterations of washing, the eggs were resuspended and transferred to a sterile 25 cm^2 culture flask with vented cap, containing 5 mL K-medium. The embryos were incubated at 20 ℃ overnight in the absence of food, which allowed the hatching of eggs but prevented further development, yielding age synchronized L1 stage nematodes.

Ochratoxin A exposure

Based on the reported contamination levels of OTA found in food and feeds (European Food Safety Authority, 2006; Joint FAO/WHO Expert Committee on Food Additives, 2008), the various maximum levels (ML) for OTA in various foodstuffs (0.5 – 10 µg/kg) (The Commission of the European Communities, 2006), and the uncertainty

factors (10-2000 folds) depending on different tested species (Dourson & Stara, 1983), 8 different concentrations of OTA were designed for this study: 0 (control), 1, 5, 10, 20, 40, 80, and 160 µM. Additionally, a preliminary lethality assay (shown in Figure 3.1) has been conducted to determine the exposure concentrations, such that the highest exposure concentrations would be nonlethal, but could result in reduced growth, reproduction, and locomotion. The stock solution of OTA (12.5 mg/ml) was used to make serial dilutions of stocks with DMSO (100% DMSO). The eight test concentrations were diluted from the serial dilutions of stocks, using K-medium containing OP50 (1mg/ml) (Brenner, 1974) as food source. The final concentration of DMSO used in these studies was prepared to be 1%, which is generally used and regarded as insignificant to nematode lethality (Schouest et al., 2009; Ura et al., 2002), growth (Boyd et al., 2012), reproduction (Boyd, McBride, et al., 2010), and locomotion (Risi et al., 2019). Three experiments were conducted with five replicate wells per concentration for the growth and reproduction experiments and three replicate wells per concentration for the locomotion experiment.

High-throughput assays in *C. elegans*

High-throughput assays were performed using the Complex Object Parametric Analyzer and Sorter (COPAS) Biosort instrument from Union Biometrica (Holliston, MA, USA, Figure 3.2) for growth and reproduction endpoints and the wMicroTracker from Phylum Tech (Santa Fe, Argentina, Figure 3.3) for locomotion endpoint. Both platforms allowed for automated data collection and quantification of various toxicity parameters for *C. elegans*, with the COPAS Biosort platform allowing for rapid and automated collection of flow data from a large variety of organisms (Shimko &

Andersen, 2014), while the wMicroTracker offers a convenient method for recording overall locomotor activity and viability by automatically monitor and quantify *C. elegans*, cultured in liquid media and in multi-well plates (Risi et al., 2019).

The COPAS™ Biosort instrument is a flow cytometer capable of the analysis, sorting, and dispensing of *C. elegans* using five sorting parameters: optical density of the object of interest (or extinction, EXT), axial length of the object (or time of flight, TOF), green fluorescence (500–520-nm wavelength), yellow fluorescence (535–555-nm wavelength), and red fluorescence (600–620-nm wavelength). TOF and EXT measurements are related to the age and size of *C. elegans*; both increase as *C. elegans* develop through larval stages into adults. The contents in each well are aspirated using the ReFLx module with the COPAS Biosort instrument and analyzed for the TOF and EXT of the individual nematode along with the total number of nematodes (Figure 3.4) (Pulak, 2006).

The wMicroTracker, on the other hand, records overall locomotor activity and viability of *C. elegans* cultured in liquid media and in multi-well plates. The tracking system is designed as shown in Figure 3.5. The wMicroTracker detects infrared microbeam interruptions (bins) in a fixed time lapse in order to measure population motility. This microbeam crosses each microtiter well from top to bottom and is finally sensed by a phototransistor. When nematodes pass across the infrared microbeam, a transient fluctuation in the signal received by the phototransistor is generated, and movement is detected by digital analysis of the phototransistor output (Simonetta & Golombek, 2007). The movement signals were then recorded and calculated to an average motility per condition for the user designated time interval.

Growth assay

Age-synchronized L1 stage nematodes were transferred to the sample cup of the COPAS Biosort and diluted to approximately 1 nematode/µL. Fifty L1 stage nematodes were dispensed into a 96-well plate, with each designated well containing 50 µL of K-medium. Afterwards, 50 µL of different concentrations of OTA were added into each well to a final volume of 100 µL. Each concentration group consisted of 5 replicate wells followed by two rinse wells to minimize carry-over of worms between the groups, as shown in Figure 3.6. After nematodes were incubated for 48 hours at 20 °C, TOF and EXT measurements of individual nematode were obtained using the ReFLx module of the COPAS Biosort. The TOF and EXT measurements were used to define the growth endpoint as the distribution of sizes of nematodes (Boyd, Smith, et al., 2010).

Reproduction (brood size) assay

Age-synchronized L4 stage nematodes were used in a reproduction assay. After obtaining age-synchronized L1 stage nematodes by hatching eggs in K-medium overnight in the absence of food, the nematodes were transferred to a newly OP50-seeded K-agar plate and incubated for approximately 48 hours at 20°C, yielding a synchronized L4 culture. The synchronized L4 stage nematodes were removed from the culture plates and washed three times with K-medium and then transferred to the sample cup of the COPAS Biosort. Five L4 nematodes were dispensed into a 96-well plate, with each designated well containing 5µL of K-medium. Afterward, 95µL of different concentrations of OTA were added into each well to a final volume of 100µL. Each concentration group

consisted of 5 replicate wells followed by two rinse wells to minimize carry-over of worms between the groups, as shown in Figure 3.7. After nematodes were incubated for 24 hours at 20 °C, the total number of nematodes per well was counted using the COPAS Biosort and recorded as brood size per replicate. The number of larvae produced during this period (i.e. number of non-adult nematodes) were used as an indication of OTA toxicity on nematode fecundity.

Locomotion assay

Age-synchronized L4 stage nematodes were washed off the K-agar plates with K-medium, where they were counted in 10 µL and adjusted to obtain concentration of 1 nematode per µL. Approximately 900 nematodes were dispensed into a 24-well plate, such that each designated well contained 900 µL of K-medium. The 900 synchronized *C. elegans* per well were assessed by the wMicroTracker. The wMicroTracker measured mean population motility per infrared sensor within each well as beam interruptions per hour. Before the 24-hour experiment, the nematodes were allowed to settle for an hour and their basal motility was measured, using the wMicroTracker, for 60 min to obtains a reference point for motility prior to treatment. The operation temperature was 20 °C. After the basal recoding, 100 µL of 10 × test solutions containing OP50 were added to each well to a final volume of 1000 µL. Eight concentrations of the test solutions were used: 0, 1, 5, 10, 20, 40, 80, and 160 µM. The motility of nematodes was measured by the wMicroTracker every hour for 24 hours continuously. Three experiments were conducted, and each concentration group had three replicate wells. A diagram summarizing the method used for locomotion assay is shown in Figure 3.8.

Statistical analysis

In order to calculate EC50s (the 50 percent effective concentration of a toxicant) and corresponding 95% CI (confidence intervals) for all endpoints used in this study, the concentration–response curves were modeled by fitting the mean values of parameters (TOF and EXT for growth, the number of offspring for reproduction, and mean activity for locomotion) to a four-parameter logistic regression model, also known as the Hill equation (Boyd, Smith, et al., 2010; Hill, 1910):

$$\hat{Y} = a + \frac{(b-a)}{\left[1+\left(\frac{c}{X}\right)^{d}\right]} \quad (1)$$

$\hat{Y}$ is taken to be the expected response at concentration X. a is the minimum response when concentration = 0, b is the maximum response for an infinite concentration, and c is the estimated concentration producing 50% of the maximal response, which we refer to as the EC50. Finally, d is the shape parameter that manages the steepness of the decrease, also known as the Hill slope (Gadagkar & Call, 2015).

Data analysis were performed using the statistical program R (The R Foundation, version 3.6.1. Vienna, Austria, www.r-project.org). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's t-test to determine significant difference from the control. A p-value of less than 0.05 was considered statistically significant.

Figures

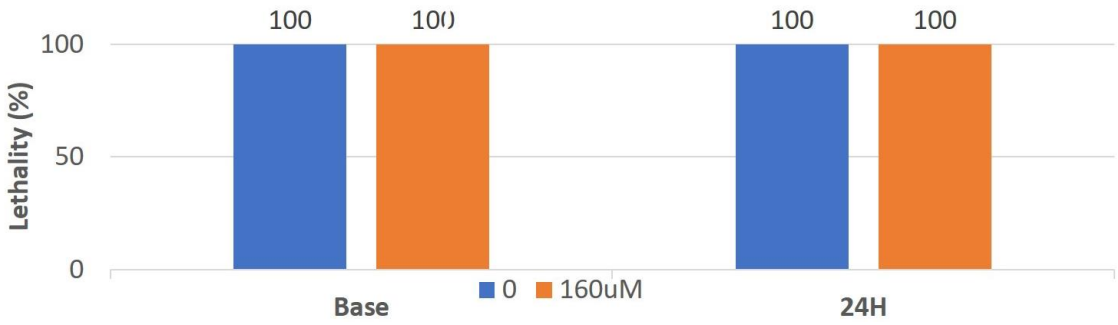


Figure 3.1 Effects on lethality of *C. elegans* exposed to OTA (0µM as control and 160µM as test concentration) for 24 hours. Ten nematodes of each well in three replicates *C. elegans* L4s were loaded at 0h (Base) and checked lethality after a 24-hour exposure of OTA.

Figure 3.2 Picture of the COPAS™ Biosort from Union Biometrica (Holliston, MA, USA) with the accompanying desktop computer and associated liquid containers. Taken from (Pulak, 2006)

Figure 3.3 Picture of the wMicroTracker from Phylum Tech (Santa Fe, Argentina)

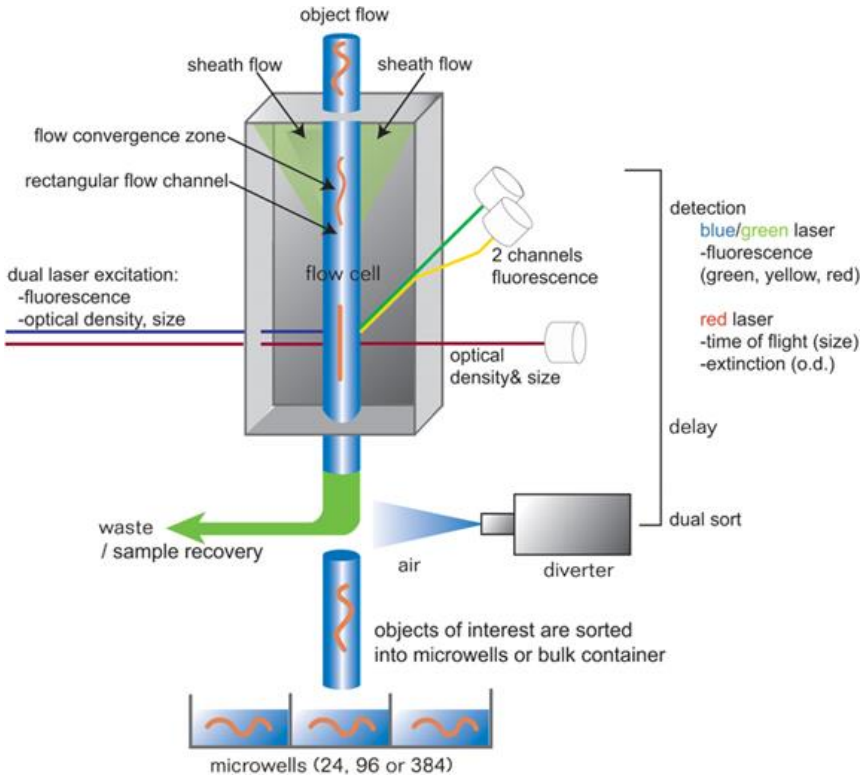


Figure 3.4 Object flow path and COPAS sorting principle. Objects flow axially through flow cell, one by one, showing the path from entry into the flow cell, orientation with the flow because of laminar flow forces, analysis in the flow cell, and sorting event. Taken from (Pulak, 2006)

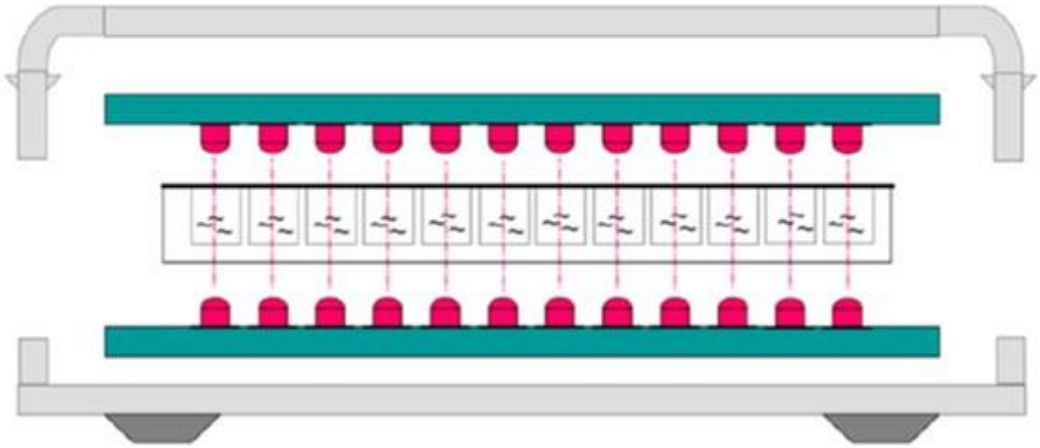


Figure 3.5 Detection of worm locomotion. The wMicroTracker detects the animal movement through infrared microbeam, light scattering. Each microtiter well is crossed by at least one infrared microbeam, scanned more than 10 times per second. The detected signal is digitally processed to calculate the amount of animal movement in a fix period of time. Taken from (Risi et al., 2019).

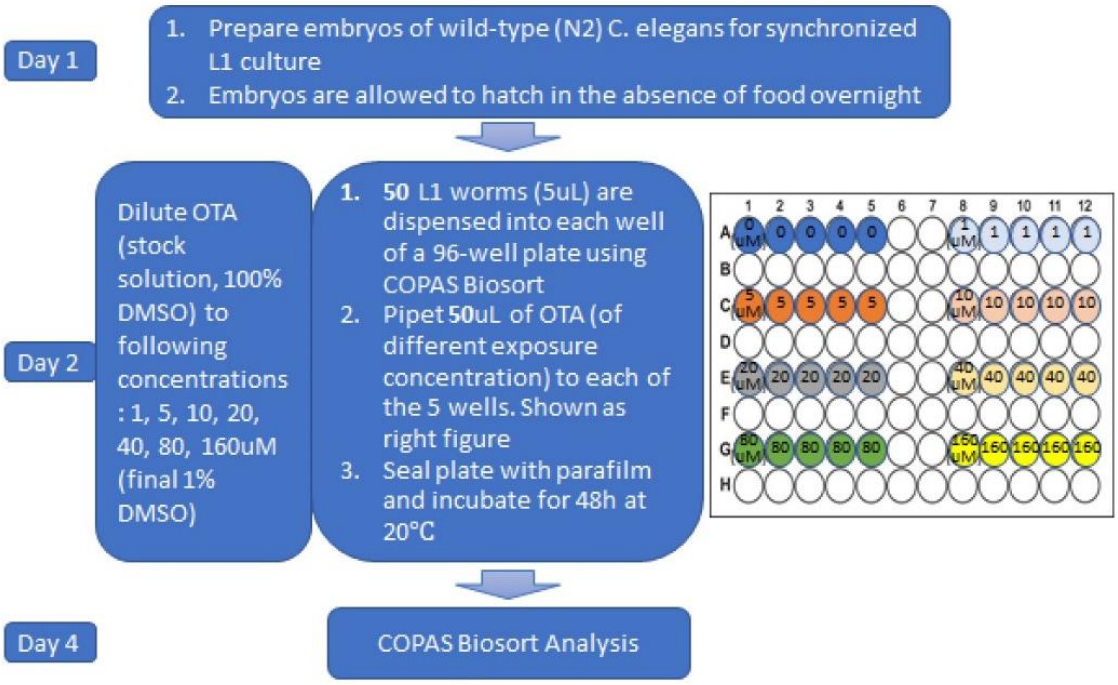


Figure 3.6 Procedure of *C. elegans* growth assay

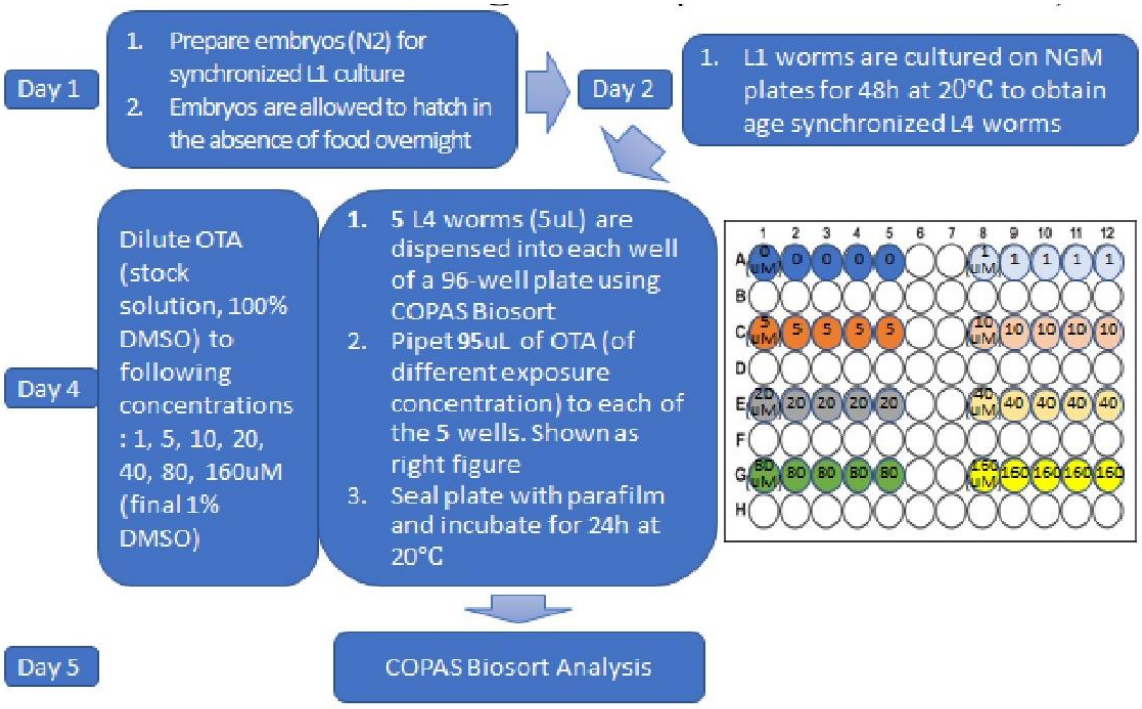


Figure 3.7 Procedure of *C. elegans* reproduction assay

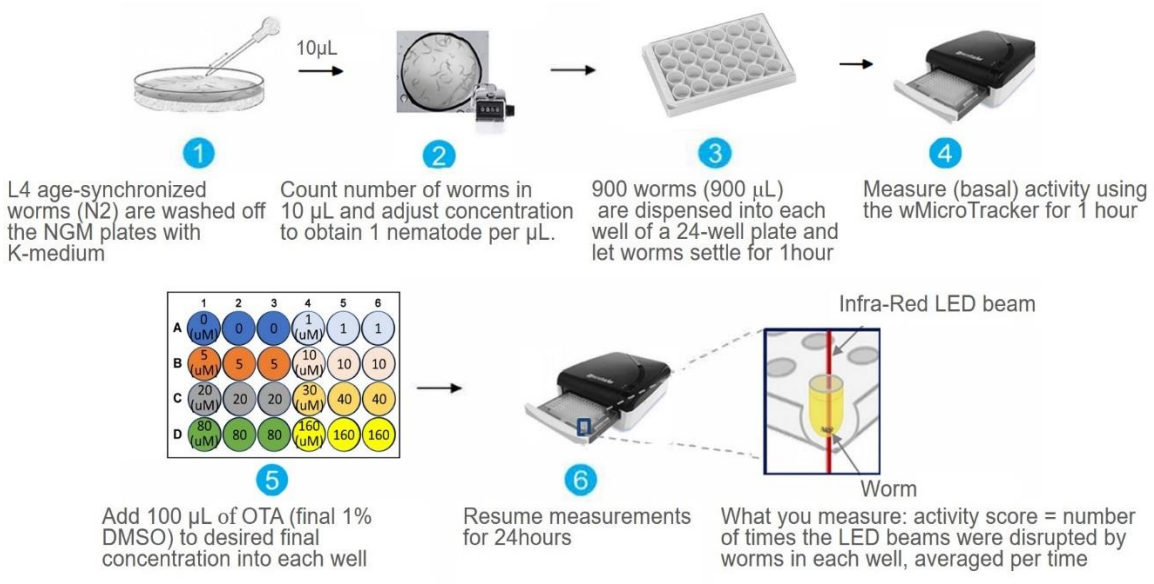


Figure 3.8 Procedure of *C. elegans* locomotion assay

CHAPTER 4

RESULTS

Toxic effects on growth

Measuring the growth of synchronized nematodes is an indicator to assess the effect of a toxicant in the development of the nematode (Tejeda-Benitez & Olivero-Verbel, 2016). A pilot study was performed to assess the concentration range for toxicity tests on growth. Based on the various maximum levels (MLs) for OTA in various foodstuffs, which were 0.5 - 10 ppb, we assumed that 100-fold of the MLs would not affect the growth of *C. elegans*; therefore, we used 1 ppm as the minimum concentration and 100 ppm as the maximum concentration for the test concentrations. The four groups for the pilot study were 0, 1, 10, and 100 ppm. As shown in Figure 4.1, a considerable growth-inhibitory effect was seen in 100 ppm ($p < 0.01$) for both mean time of flight (TOF) for axial length and mean extinction (EXT) for optical density in studied *C. elegans*. However, we could not get a concentration-response curve and EC_{50}, because there was no statistically significant data for fitting the four-parameter logistic regression to the evaluated TOF and EXT. To get a concentration-response curve, a more variable concentration range was needed in this study, so the endpoint of growth toxic effect was eventually evaluated at eight different concentrations of OTA in 0, 1, 5, 10, 20, 40, 80, and 160 μM with three independent experiments. Using the COPAS Biosort, growth was defined by the TOF and EXT measurements as the change in the distribution of sizes of

nematodes (Boyd, Smith, et al., 2010). As shown in Figure 4.2, OTA negatively affected the growth of the nematode with a concentration-dependent manner in both measured TOF and EXT. Following the 48-hour exposure, OTA at 80 and 160 μM caused significant ($p < 0.001$) growth-inhibitory effects on *C. elegans*, lowering the values to 64.0% and 35.0% in TOF and 56.2% and 24.0% in EXT, compared to the untreated control, respectively. To estimate EC_{50} values, the Hill equation (Eq. (1)) was fit as a function of OTA concentration to the estimated mean TOF and EXT. The EC_{50}s were estimated as 82.51 μM in TOF and 79.78 μM in EXT; the EC_{50} values with 95% CI were listed in Table 4.1.

Toxic effects on reproduction

Brood size (the number of offspring) produced for a 24-hour exposure to OTA is an indicator to evaluate whether OTA can affect reproduction of the nematodes, placing exposed L4 nematodes onto K-agar plates. Brood size was counted and compared with a control group. In this study, the brood size was calculated by counting the total number of larvae after a 24-hour exposure to OTA and dividing the counted total number by the number of adult nematodes (five nematodes), using the COPAS Biosort. Five age-synchronized L4 nematodes were dispensed into a 96-well plate, with each well containing different concentrations of OTA. After incubation for 24 hours at 20 ℃, the total brood size was measured per replicate. A pilot study of reproduction was performed to identify a concentration range, same as the growth assay. The four concentration groups for the pilot study were 0, 1, 10, and 100 ppm. As shown in Figure 4.3, the toxic effect of OTA on reproduction in the pilot study was found, including significant

reduction in the brood size at all exposed concentration groups (1, 10, and 100 ppm); reaching only 49.9%, 45.6%, and 42.1% offspring of the untreated control group, respectively. Based on the result of the pilot study, we have set the eight concentrations of OTA in 0, 1, 5, 10, 20, 40, 80, and 160 μM with three independent experiments. Figure 4.4 presents the representative experiment showing the concentration-response curve for the effects of OTA on brood size for wild-type N2 nematodes. To estimate EC_{50} values, the Hill equation (Eq. (1)) was fit as a function of OTA concentration to the estimated brood size. Following 24-hour exposure of OTA to the nematodes, as concentration increases, the brood size significantly decreases for almost all tested concentrations, as compared to the untreated control. The EC_{50} value of reproduction was estimated as 8.10 μM (shown in Table 3.1).

Toxic effects on locomotion

Toxic effects on a toxicant to the locomotion of nematodes have been related to a neurotoxicity which can be evaluated based on several criteria, such as head thrash, body bend frequency, and basic movements. In this study, we used basic movements of nematodes as the endpoint of locomotion and the wMicroTracker to record overall motility in *C. elegans*. The experiment was repeated three times with three replicate wells per concentration. Eight concentrations of OTA, same as the growth and reproduction assays, were applied to each well of a 24-well plate, treated with 0, 1, 5, 10, 20, 40, 80, and 160 μM.

As shown in Figure 4.5, the motility of nematodes decreased over time in all concentration groups; however, the decreasing slope varied with concentrations. Indeed,

as OTA concentration increased, the motility declined with a gradual slope, which meant that worms treated with higher concentration of OTA showed higher motility than the ones treated with lower concentration of OTA. The motility of the control group dropped quickly from 100% to 47% during first 12 hours, followed by a quite flat slope from 47% (13 hours) to 44% (24 hours). On the contrary, the motility of the highest concentration exposed (160 μM) group was much more gradual from 100% to 69% during first 12 hours as compared to the control, but the motility gradually increased from 67% (13 hours) to 78% (24 hours). After a 24-hour exposure, the untreated control worms showed the lowest motility as compared to other OTA-treated groups, with a 44% of the basal measurement; however, the motility of worms in the maximum concentration, 160 μM, decreased to a 78% compared to the basal measurement, and showed the highest motility.

Since the motility curves over time were difficult to compare the motility data in different concentration groups, Figure 4.6 presented motility graphs at four-hour interval from 4 to 24 hours, which are normalized by the motility of the untreated control, plotted against the different OTA concentrations (0, 1, 5, 10, 20, 40, 80, and 160 μM). The motility of worms increased over time along with the exposed concentration of OTA. At 4 hours, the motility of worms in all concentrations were not significantly different from that in the untreated control. However, at 8 and 12 hours, the motility of worms increased with a concentration-dependent manner in worms treated with 20, 40, 80, and 160μM OTA, and were significantly higher than that in the untreated control. Similarly, at 16, 20, and 24 hours, populations of worms exposed to a OTA concentration range from 1 to 160 μM showed a concentration-dependent increase in the motility with significantly higher

motility found in worm groups treated with 10, 20, 40, 80, and 160 µM OTA than that in the untreated control group.

Tables

Table 4.1 EC_{50} values of OTA in growth and reproduction assays with *C. elegans*

Assay		EC_{50} (µM)	95% CI (µM)
Growth	TOF	82.51	44.26 – 120.77
	EXT	79.78	56.95 – 102.61
Reproduction		8.10	6.75 – 8.44

Figures

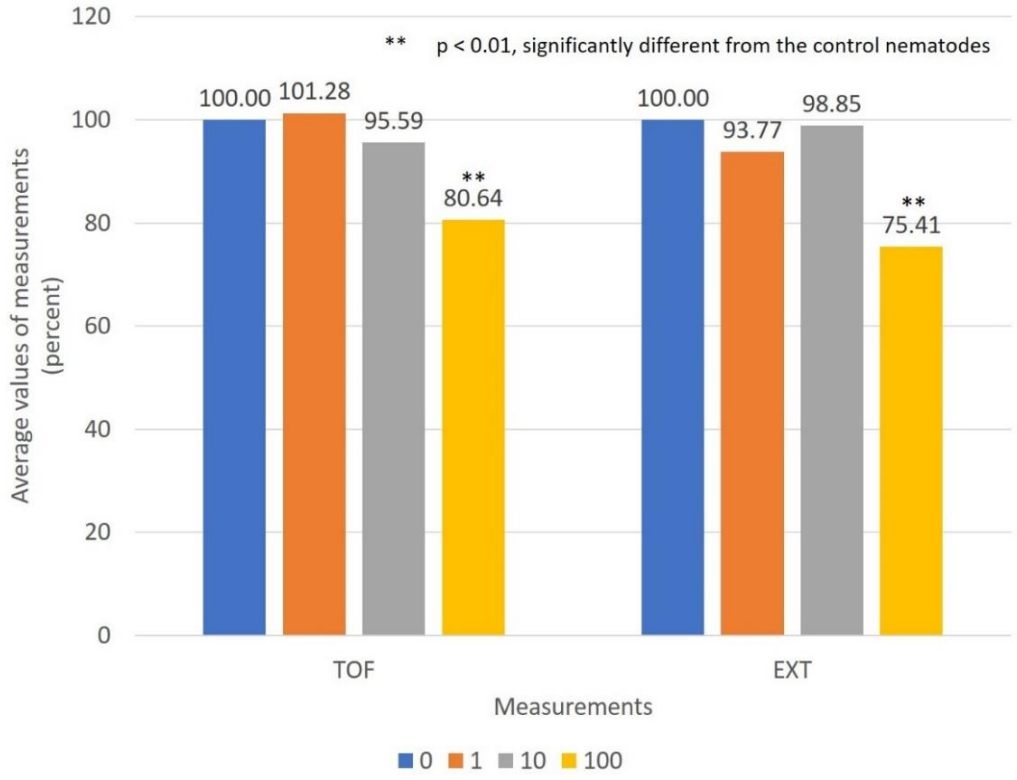
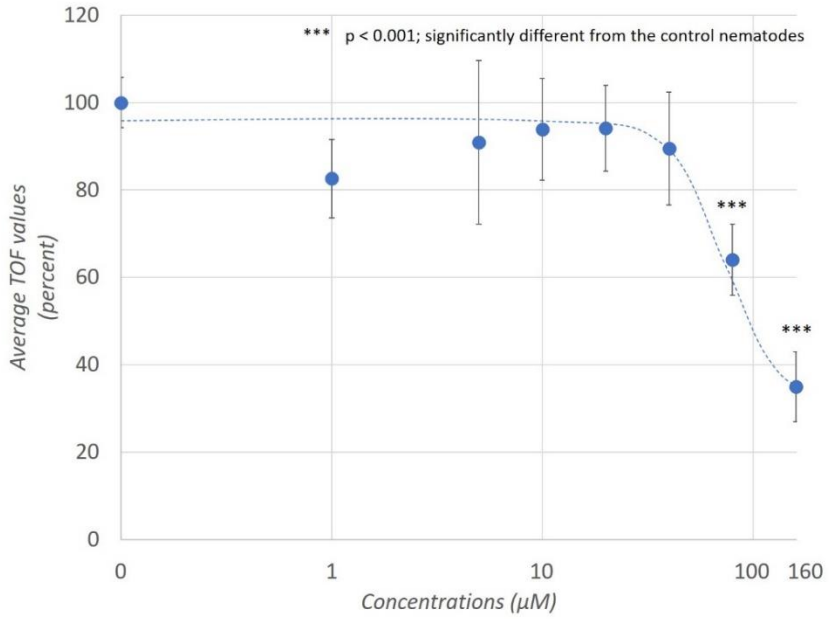


Figure 4.1 Toxic effects on growth of *C. elegans* exposed to OTA at 0, 1, 10, and 100ppm in a pilot study. TOF and EXT measurements were recorded for evaluating the growth.

(a)



(b)

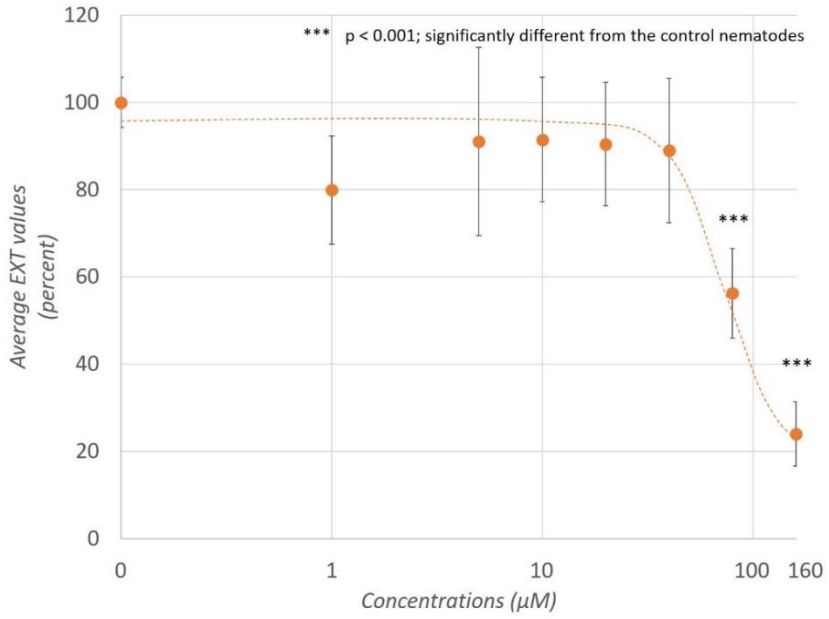


Figure 4.2 Toxic effects of OTA on *C. elegans* growth. This illustrates the effects of 48 h exposures on (a) the mean TOF and (b) EXT values at eight concentrations of OTA in *C. elegans* growth. To estimate EC_{50} values, the Hill equation (Eq. (1)) was fit as a function of OTA concentration to the estimated mean TOF and EXT.

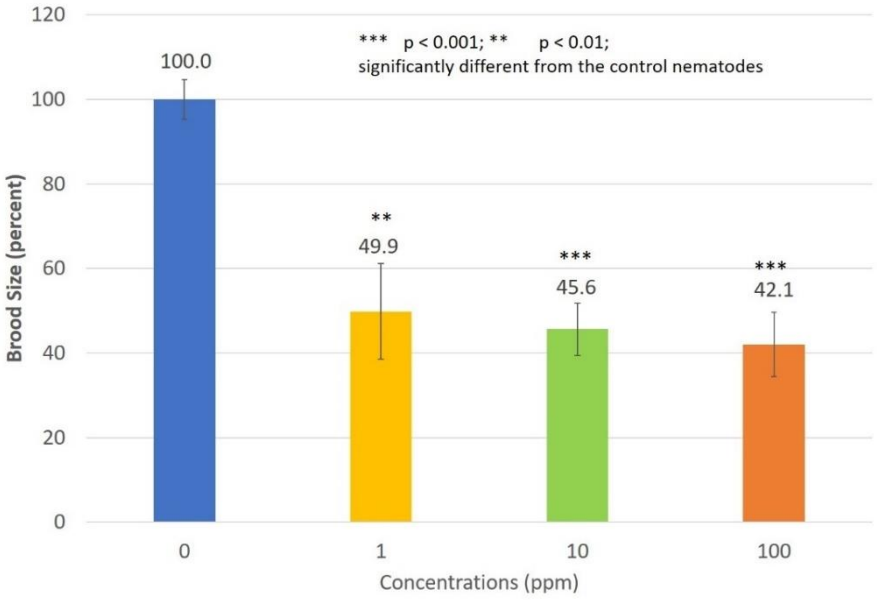


Figure 4.3 Toxic effects on reproduction of *C. elegans* exposed to OTA at 0, 1, 10, and 100ppm in a pilot study. Brood size was measured for evaluating the reproduction.

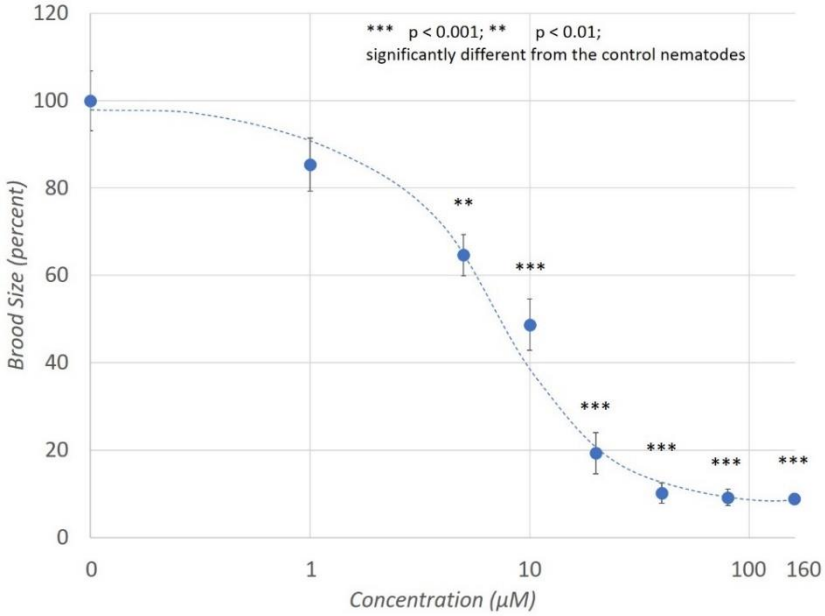


Figure 4.4 Toxic effects of OTA on *C. elegans* reproduction. This illustrates the effects of 48 h exposures on brood size at eight concentrations of OTA in *C. elegans*. To estimate the EC_{50} value, the Hill equation (Eq. (1)) was fit as a function of OTA concentration to the estimated mean brood size.

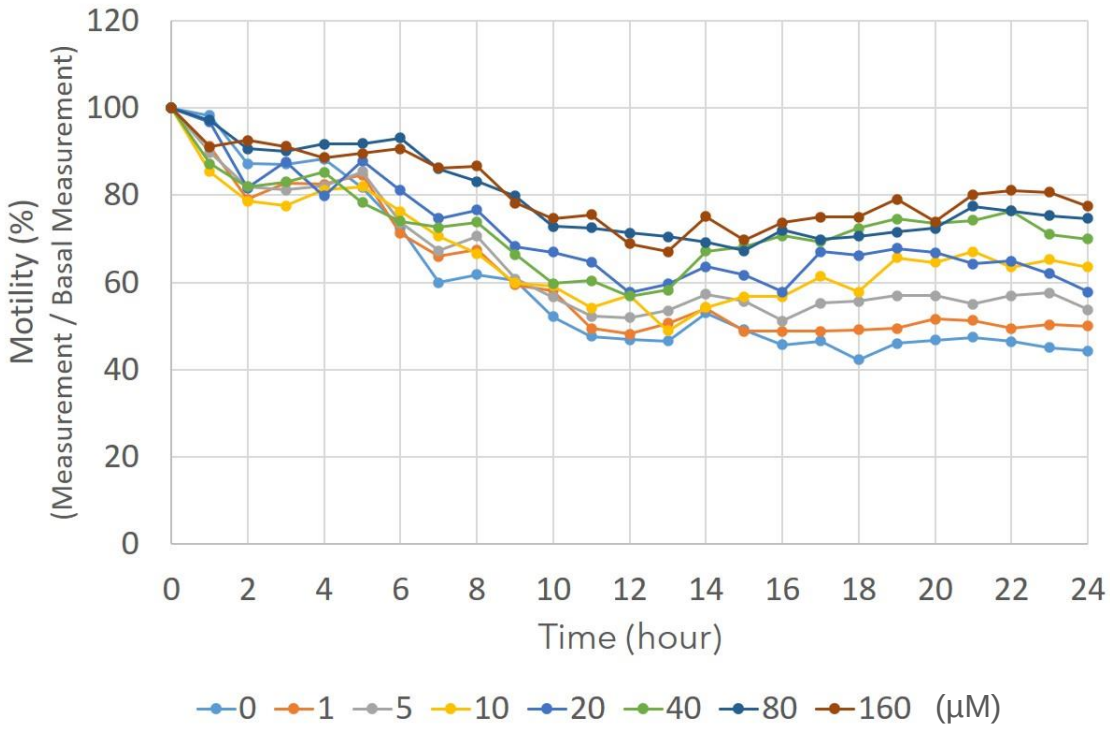


Figure 4.5 The graph represented the mean motility of the nematodes over time in three replicate wells of each concentration. The vertical axis shows values of the motility, while the first measurement is for the basal motility. The horizontal axis shows the time for the measurement. The L4 synchronized nematodes and the 24-well plate were used in the experiments. 900μl worm suspension (900 worms), containing OTA with different concentrations of 0 μM (blue), 1 μM (red), 5 μM (gray), 10 μM (yellow), 20 μM (aqua), 40 μM (green), 80 μM (deep blue), and 160 μM (brown), were transferred to separate wells respectively. The motility was measured by the wMicroTracker every hour for 24 hours.

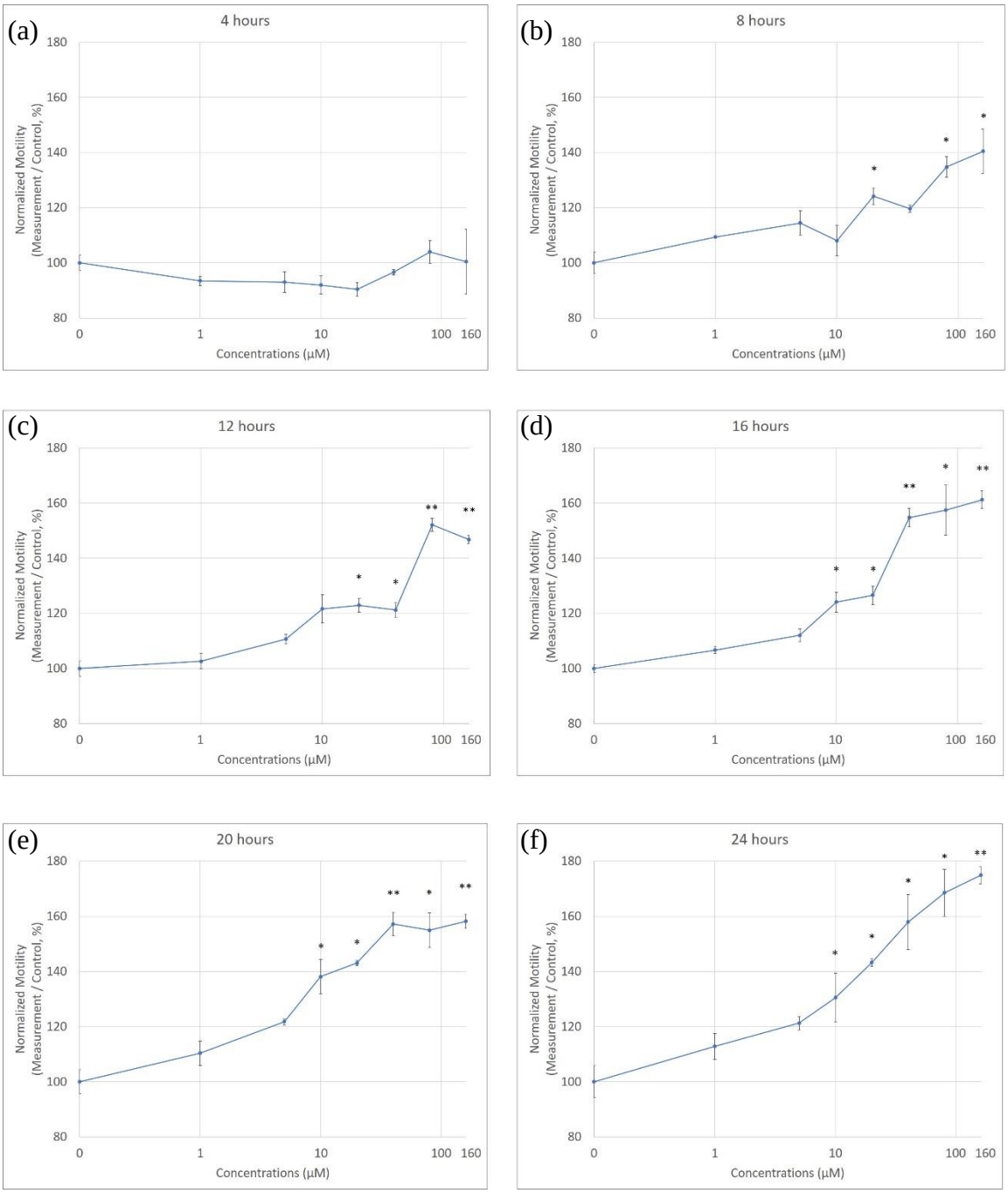


Figure 4.6 OTA effects on *C. elegans* motility: four-hour interval graphs from 4hours to 24hours. (a) at 4 hours, (b) at 8 hours, (c) at 12 hours, (d) at 16 hours, (e) at 20 hours, and (f) at 24 hours. The motility was normalized by the motility of the untreated control and plotted against the different concentrations (0, 1, 5, 10, 20, 40, 80, and 160 μM). ** ($p < 0.01$) and * ($p < 0.05$) represent significantly different from the untreated control.

CHAPTER 5

DISCUSSION AND CONCLUSION

Discussion

Using *C. elegans* in toxicological research provides numerous advantages that have been well reviewed in detail (Anderson et al., 2001; Boyd et al., 2012; Hunt, 2017; T. Kaletta & M. O. Hengartner, 2006; Leung et al., 2008; Meyer & Williams, 2014; Williams et al., 2000; Williams & Dusenbery, 1988). Traditionally, *C. elegans* model was used for much of the early work to identify metal toxicity and used lethality as the major endpoint (Williams & Dusenbery, 1988). In recent years, a lot of studies have used the *C. elegans* model with a diverse set of toxicants, and more complicated sub-lethal endpoints, including growth, reproduction, feeding, and locomotion, have been developed (Anderson et al., 2001; Hunt, 2017; Tejeda-Benitez & Olivero-Verbel, 2016). The sub-lethal endpoints may serve as an important contribution on evaluating environmental toxicants. In addition, these types of endpoints may be used as an alternative method for mammalian models (Hunt et al., 2018), because of a number of strength: the nematode shares basic biological functions with mammals, such as DNA processes; the structure and function of *C. elegans* nervous system are a simplified human version, embryogenesis and organogenesis pathways of *C. elegans* are well defined and invariant, and *C. elegans* possesses microsomal enzymes that perform biotransformation reactions (Meyer & Williams, 2014; Williams et al., 2000). Due to these unique features in *C.*

elegans model, the nematode has been massively applied to toxicological fields in recent years, but few studies have investigated it for toxicological effects of foodborne mycotoxins. Yang *et al*. (Yang et al., 2015) evaluated the multiple toxic endpoints of common mycotoxins, Aflatoxins B_1 (AFB_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), T-2 toxin (T-2), and zearalenone (ZEA), in *C. elegans* model, including lethality, toxic effects on growth and reproduction, as well as influence on lifespan. They suggested that *C. elegans* model may serve for evaluation of toxic effects of mycotoxins, especially for assessing developmental and reproductive toxic effects. In another study, the progeny production and development rates of the nematode were significantly reduced when treated with AFB1 at concentrations of 10, 30, and 90 µM (Feng et al., 2016).

To our knowledge, the present study is the first time that the toxic effects of OTA on *C. elegans* have been investigated. We assessed OTA toxicity in *C. elegans* with commonly used sensitive indicators, including growth, reproduction, and locomotion. Our results showed that growth and reproduction were significantly decreased upon the exposure to OTA, and these effects seemed to be concentration-dependent. The EC_{50} of reproduction was 8.10 µM, which was much lower than the EC50s of growth, 82.51 µM in TOF and 79.78 µM in EXT. Yang et al. (Yang et al., 2015) also found that EC_{50}s of reproductive effects of mycotoxins, including AFB_1, DON, FB_1, T-2, and ZEA, in *C. elegans* with much lower concentrations than those on the growth effects. These results suggested that *C. elegans* is a much more sensitive model for testing reproductive toxic effects of mycotoxins, as compared to the toxic effects on growth.

OTA has been shown to cause growth retardation in rats (Munro et al., 1974), turkeys (Dwivedi & Burns, 1985), and chickens (Galtier et al., 1976). In this study,

exposure to different concentrations of OTA led to decreased growth in *C. elegans*, same as the growth retardation in the results of other animal studies; it implied that *C. elegans* might be an appropriate candidate for further testing the growth toxicity of OTA.

For the reproduction assay, the average number of offspring after a 24-hour OTA exposure was significantly reduced at all tested concentrations, compared to the number of offspring of the control group with a concentration-dependent manner. OTA is known to cause various reproductive effects in laboratory and domestic animals such as decreased fertility or teratogenicity. In female Institute of Cancer Research (ICR) mice, 10.0 μM of OTA exerted injurious effects on the oocyte maturation rate, as well as fertilization and embryonic development (Huang & Chan, 2016). When male albino mice were treated with OTA (250 and 500 μg/ml per day for 45days, orally), OTA exposure altered sperm count, sperm motility, sperm viability, and fertility rate in a dose-dependent manner (Chakraborty & Verma, 2009). As with other animal test results of reproduction, the result of the reproduction test in this study suggests that *C. elegans* may be an appropriate candidate for further testing the reproductive toxicity of OTA.

For locomotion assay, populations of *C. elegans* exposed to a concentration range (1–160 μM) of OTA showed a time-dependent decrease in the motility of worms. In the process of time, the motility of worms treated with higher-concentrated OTA was generally higher than the motility of worms treated with lower-concentrated OTA. The concentration-response graphs in every four-hour interval showed that exposure of OTA induced locomotion-excitatory effects in *C. elegans*, compared to the untreated control with a concentration-dependent manner. Since recent studies have indicated that OTA may affect the neuronal system (Paradells et al., 2015; Sava et al., 2006), many research

groups have focused on the mechanism of neurotoxic action of OTA *in vitro* and *in vivo*, evidenced by its potent action as a function of time and concentration (Sava et al., 2007; Zhang et al., 2009). Despite the efforts, the underlying molecular mechanisms for OTA neurotoxicity are not fully understood. Therefore, based on the locomotion result of this study, further studies should be conducted to verify which mechanisms of neurotoxic action in OTA may induce locomotion-excitatory effects in *C. elegans*.

Up to now, no study has been conducted on assessing the toxicity of OTA in *C. elegans*. The present study is the first to report the toxic effects of OTA in the nematodes using high-throughput assays. Our results showed that OTA displayed toxic effects on growth, reproduction, and locomotion in wild type *C. elegans*. Data obtained from this study may provide useful information and knowledge on evaluation of toxic effects of OTA using *C. elegans* model, especially for assessing growth, reproductive, and locomotion toxic effects of OTA exposure in humans and animals.

Conclusion

OTA has become an important study topic in recent years, because of its renal carcinogenicity in several animal species (Duarte et al., 2011) and its widespread and continuous exposure to humans (Scott, 2005). This study evaluated the toxicological effects of OTA to *C. elegans* based on multiple toxic endpoints, including growth, reproduction, and locomotion. Using high-throughput assays (the COPAS Biosort and wMicroTracker), toxic effects of OTA in *C. elegans* have been evaluated in response to multiple concentrations of OTA. Both high-throughput assays were accurate and reliable in evaluating OTA toxicity. Our results showed that growth and reproduction in *C.*

elegans were significantly reduced upon the exposure to OTA, and these effects are concentration-dependent. In addition, the result of the locomotion experiment for exposure of OTA to *C. elegans* displayed a time-dependent decrease in the motility of worms, but, as time went on, a concentration-dependent increase in the motility of worms was found as compared to the untreated control. These findings indicated that toxicological evaluations of OTA in *C. elegans* presented similar results in other studies of mycotoxins in *C. elegans*. So far, no study has reported the toxic effects of OTA in *C. elegans*. Our research, therefore, sheds some light on this field. This thesis research suggested that *C. elegans* model can serve as a good model organism for evaluation of toxic effects on OTA and mechanistic studies of OTA-induced adverse health effects.

CHAPTER 6

FUTURE DIRECTION

OTA frequently co-occur with other mycotoxins produced by *Aspergillus* and *Penicillium* genera. Among different mycotoxins, a combination of OTA and citrinin (CIT) is one of the most frequently occurring mixtures in a wide variety of food and feed commodities. Several studies have shown that both OTA and CIT cause several toxic effects to animals and humans, but few studies have been performed in the combined toxicity of OTA with CIT and associated health risks. Therefore, future studies should be conducted on assessing the toxic effects on the combination between OTA and CIT in *C. elegans* with multiple endpoints.

The phenotypical endpoints, centered on growth, reproduction, and locomotion, were used in this thesis project, and we found the concentration-dependent results. Using these results, future studies should focus on better understanding the mechanistic pathways of OTA toxicity in *C. elegans*. Exposing *C. elegans* to OTA causes concentration-dependent growth and reproduction inhibition, so we need to find which mechanisms of OTA-induced toxicity play key roles in toxic effects on the development and reproduction of *C. elegans*. In addition, further studies should be conducted to identify which mechanisms of neurotoxicity would lead to excitatory effects on locomotion in *C. elegans*. Specifically, in the locomotion experiment, we found that the motility of nematodes decreased over time in all concentration groups, but the motility of OTA-treated nematodes declined with a more gradual slope than the untreated control.

Therefore, future studies may design a locomotion experiment in which the motility of worms is measured with different start times of OTA exposure, such as different developmental stages of the nematode (L1, L2, L3, L4 and adult) or morning and afternoon. In addition, there are other ways to vary OTA exposure duration for each start time, such as 24, 48, and 72 hours exposure.

So far, neither the potential of OTA to cause toxic effects on growth, reproduction, and locomotion in humans nor the mechanism of OTA to induce those toxicity in humans is fully understood. One of the goals for OTA toxicity tests in *C. elegans* is their eventual application in the prediction of human toxicological response. Future results of *C. elegans* toxicological studies, including the mechanistic pathways of OTA toxicity, may be used to predict human toxicity.

CHAPTER 6

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