THE EFFECT OF SUBCUTANEOUSLY INJECTED CADMIUM ON OOGENESIS AND PROGENY IN XENOPUS LAEVIS: APPLICATIONS FOR THE DEVELOPMENT OF A REPRODUCTIVE TOXICITY ASSAY

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for Care and Use of Laboratory Animals (NRC 1996) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Chapter I

LITERATURE REVIEW

Introduction

Cellular and molecular processes differ among the various stages of an organism's life cycle. These differences can cause a varied sensitivity to xenobiotics, where some life stages may be more sensitive because of specific cell receptors or differences in metabolism. Therefore, it is important that toxicity screening assays account for the different stages of the life cycle, especially the most sensitive stages such as within reproduction and development. Reproduction and development are complex and specialized processes that begin with gametogenesis, continue through fertilization and embryogenesis, and end in the sexual maturation of the organism. In designing a reproductive toxicity assay it is critical to assess toxicity at the stages in an animal's life cycle when sensitivity to toxicants occur (Mattison and Thomford, 1989). For example, gametes can be affected by toxicants at levels well below those of adults. Many current reproductive and developmental toxicity tests may be missing significant toxicity by excluding the effects on gametes and full life cycle studies are not practical in most vertebrates due to their long life spans.

The South African Clawed Frog (*Xenopus laevis)* is well suited for reproductive toxicity studies. The eggs of females are derived from stem cell oogonial populations that can continually generate a new cohort of oocytes (Gilbert and Raunio, 1997). The oogonia replicate by mitosis and then undergo oogenesis to produce haploid oocytes. During oogenesis, oogonia develop into primary oocytes, which are arrested in meiotic prophase I (diplotene) (Gilbert and Raunio, 1997). Upon oocyte maturation, gonadotropic hormones trigger the resumption of meiosis to metaphase II where the oocyte is once

again arrested until fertilized. Interruption, such as by a xenobiotic, during meiosis can result in aneuploidy as well as other chromosomal aberrations and mutations (Mailhes et al., 1986).

A xenobiotic that shows no effect upon the adult organism may very well affect the progeny of that animal. It may pass from egg to embryo unnoticed possibly by bioaccumulating in the ovary, damaging the developing oocyte and then passing from parent to progeny. It is, therefore, important to incorporate next generational testing in a reproductive toxicity assay.

The heavy metal cadmium (Cd) is suitable for reproductive studies with *Xenopus*. It has been widely researched and has been demonstrated to be teratogenic in many organisms including amphibians (Webster, 1990). Cadmium was first shown to be a teratogen by Ferm and Carpenter (1967) in golden hamsters. Cadmium can cause sister chromatid exchanges in human lymphocytes (Saplakoglu and Iscan, 1998) and mitotic aneuploidy in hamster primary embryonic cells (Natarajan et al., 1993). Cadmium induces a wide variety of malformations affecting all organ systems as well as causing intrauterine growth retardation and prenatal death in mammals (Webster, 1990).

The primary objective of my research study was to investigate the effect of Cd on oogenesis and progeny of *Xenopus laevis*. Although numerous toxicity studies have been conducted using *Xenopus*, testing has concentrated on the effects of a xenobiotic either on adults or directly on the embryos. Little is known about the effects of toxicants on *Xenopus* oogenesis or on the progeny of exposed adults. Cadmium is a known teratogen and carcinogen (Eisler, 1985; Webster, 1990). It has been shown to be an embryotoxicant that causes reduced fecundity in amphibians and mammals (Sunderman et al., 1991; De et al., 1993).

I predicted that Cd would accumulate in the ovary of *Xenopus* and disrupt the process of oogenesis. Subsequently, I hypothesized that fertilization and development of the progeny from exposed adult females would be affected.

The second objective of my thesis research was to develop a preliminary protocol of a reproductive toxicity assay, using *Xenopus*, that could be utilized in conjunction with other toxicity tests such as FETAX (Frog Embryo Teratogenesis Assay - *Xenopus*) and other assays of immunotoxicity, genotoxicity, and neurotoxicity. The goal of this phase of the assay development was to test a single reproductive toxicant, re-evaluate the length and route of exposure, and examine the endpoints to ensure that relevant information was being collected.

LITERATURE REVIEW

A. Toxicity Testing

In order to achieve a thorough assessment of hazards to an organism, toxicity testing must take into account the different stages in the organism's life cycle. Testing should, therefore, incorporate all aspects of toxicity including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity. In addition, reproductive toxicity tests should examine the effects of xenobiotics on gametes.

Historically, much of reproductive toxicity testing had centered primarily on the male reproductive system. Concern with male reproduction has escalated following the discovery of declining sperm counts over the past 50+ years. Research has been conducted on the processes of spermatogenesis as well as changes in testes morphology (Mori et al., 1992; Majumder and Kumar, 1995; Llobet et al., 1995; Pant et al., 1995; Boockfor and Blake, 1997).

Female reproductive toxicity was not vigorously studied until recently and as such there are very few validated assays that test toxicant effects on the female reproductive, embryonic and developmental cycles (Kamrin et al., 1994). The preponderance of female reproductive toxicity tests are conducted with mammals, specifically on events occurring after fertilization (Levin and Miller, 1980; Rice, 1981; Wier et al., 1990; De et al., 1993). For example, researchers have examined the effects of toxicants on post-fertilization events such as the implantation of embryos (Rice, 1981; Eisenmann and Miller, 1994) and interaction between mother, placenta and fetus (Wier et al., 1990). Additionally, the cellular processes such as gap junction communication during muscular contractions in the uterus have been reviewed by Kamrin et al. (1994). Paksy et al. (1997) showed that Cd interfered will cell-cell junctions and cell adherence in rats. Studies have also been conducted with vertebrates other than mammals. Michibata et al. (1987) have examined the survival of teleost fish eggs exposed to varying toxicant concentrations, the amount of toxicant accumulated by the egg, and the location of the toxicant within the egg and surrounding membranes.

The endpoints that can be evaluated in a reproductive toxicity test are numerous. Adult animals can be exposed and their gonads and/or gametes examined for morphological changes (Lohiya, 1976; Mattison et al., 1983; Sato and Koide, 1984; Jarrell et al., 1988; Toth et al., 1992; Domingo, 1995). The effect of the toxicant on fecundity can be examined by exposing adult females (Kramer et al., 1998). Transgenerational effects on the progeny of exposed adults can be assayed using the endpoints from FETAX. Endpoints would include mortality, malformation, growth inhibition, behavior, pigmentation, locomotion and feeding ability. Additionally, a 96-h LC50 (mortality) and 96-h EC50 (malformation) can be determined by probit analysis of the data collected from FETAX. A teratogenic index (TI) can then be calculated by

dividing the LC50 by the EC50. The TI is a comparison of observed toxicities between an adult and embryo and is a measure of developmental hazard. A compound with a TI < 1.5 is interpreted to have a low developmental hazard.

Developmental toxicity to embryos has also been an endpoint that has, and continues, to be the subject of many studies (Perez-Coll et al., 1985; Woodall et al., 1988; Antonijczuk et al., 1995; Herkovits et al., 1997; Key et al., 1998). In these tests fish or amphibian embryos are usually exposed to a toxicant (generally for 96 hours) and then assayed for developmental abnormalities. For example, FETAX, developed by Dumont et al. (1983) and standardized by Bantle et al. (1989)(ASTM, 1992) is widely used to screen for teratogenic risks from chemical agents. The embryotoxicity of metals to *Xenopus* has been widely tested using FETAX (Sunderman et al., 1991; Sunderman et al., 1992; Sunderman et al., 1995a).

The bulk of developmental toxicity tests investigate post-fertilization effects of xenobiotics, however, significant toxicity may be overlooked by not utilizing reproductive toxicity tests that examine the gametes and gametogenesis. Thus, the development of a reproductive toxicity test that examines the effect of toxicants on the developing gamete is necessary.

B. The importance of female toxicity testing

The ovary is key to successful reproduction through the production of gametes and hormones. Ovarian function is integrated with other organ systems by the central nervous system through the hypothalamus and pituitary gland (Mattison and Schulman, 1980). Some compounds may impair reproduction by inhibiting female hormonal function. Hormonal interactions between the hypothalamus, pituitary gland and follicle cells in the ovary regulate numerous activities such as vitellogenesis. Vitellogenesis

entails both the synthesis of vitellogenin by the liver and its uptake by oocytes (Sumpter and Jobling, 1995) through micropinocytosis. Vitellogenesis can be inhibited when organisms are exposed to certain compounds (Lessman and Habii, 1987; Povlsen et al., 1990; Olsson et al., 1995). Effects of toxicants that inhibit or increase vitellogenesis may be determined by examining the oocytes at these vitellogenic stages. Oocytes can be easily removed from the ovary either by using collagenase or manually (Smith et al., 1991). Lipovitellin 1, a yolk protein derived from vitellogenin, has been found to bind Cd^{2+} (Sunderman et al., 1995b). Toxicants such as Cd that are able to bind to these yolk proteins may cause a disruption in oogenesis.

The amount of follicle stimulating hormone (FSH) in mammals may also be a valuable biomarker in assessing the number of oocytes exposed to a toxicant (Jarrell et al., 1988). FSH is required for oocyte maturation. Luteinizing hormone (LH) from the pituitary provides the physiological trigger for ovulation and germinal vesicle breakdown (GVBD) in female mammals. Agents that compromise this increase in LH can function as reproductive toxicants (Goldman et al., 1991).

Lead, benzo(a)pyrene and Aroclor 1254 have been shown to impair ovarian growth and significantly decrease plasma testosterone levels in female Atlantic Croakers (Thomas, 1988; Thomas, 1990). Lead and Aroclor 1254 decreased the secretion of gonadotropin (GtH) and lead and benzo(a)pyrene reduced the amount of circulating 17β-estradiol. Gonadotropic hormones secreted by the pituitary in response to hormones released by the hypothalamus stimulate follicle cells to secrete estrogen. Estrogen in turn directs the liver to synthesize and secrete vitellogenin. Thus, decreased GtH can result in a disturbance of vitellogenin synthesis. Xenobiotics, therefore, can alter the activity of the entire hypothalamus-pituitary-gonadal-liver axis (Thomas, 1990)

Some toxicants destroy the oocytes. These ovotoxicants can lead to decreased reproductive capacity and/or premature ovarian failure. Polycyclic aromatic hydrocarbons (PAHs) have been shown to destroy oocytes in mice (Mattison et al., 1983). Once distributed into the ovary, the parent hydrocarbon is metabolized by ovarian enzymes to the ovotoxic reactive intermediates.

Effects of toxicants upon oocytes and the ovary can be ascertained without using advanced molecular techniques. Oocytes from exposed adults can be removed and assessed for morphological changes, delay in oocyte maturation and other endpoints (Pramoda and Saidapur, 1986; Den Besten et al., 1989; Brown et al., 1994).

C. The study animal - *Xenopus laevis*

Amphibians represent an ideal model of vertebrate development (Muller, 1997) and *Xenopus laevis* is well suited for female reproductive studies because of its fecundity and continuous asynchronous oogenesis. A new reproductive cycle can be initiated during any season by administration of hormones (Dumont, 1972). Additionally, an extensive knowledge base has been developed through its use for research in developmental and molecular biology. The study of *Xenopus* oogenesis has led to our understanding of the molecular mechanisms of human oogenesis (Muller, 1997).

Amphibian oogenesis begins with the development of oocytes from oogonia (Gilbert and Raunio, 1997). The ovary of *Xenopus laevis* consists of several lobes containing oocytes at varying stages of development (Dumont, 1972; Smith et al., 1991). Dumont (1972) classified these stages, based mainly on size, as stage I (smallest) through stage VI (largest). Stage VI oocytes are ready to be ovulated. A new reproductive cycle can be initiated in the laboratory at any time with the injection of human chorionic gonadotropin (hCG). Dumont (1972) found that after animals were injected with hCG, it

took approximately 5-7 weeks for a new population of mature stage VI oocytes to develop. Keem et al. (1979) were able to determine the oocyte growth rate from stage III to Stage VI in laboratory maintained females. They estimated that in non-stimulated (non-hCG-induced) animals it took approximately 16-24 weeks, while it took 9-12 weeks in hCG-stimulated frogs. Overall, the entire process of oogenesis, from stage I to stage VI takes approximately 8 months (Smith et al., 1991).

At the onset of oogenesis, the oocytes begin meiosis but are arrested in the diplotene stage of prophase I. During the diplotene stage, the chromosomes become highly extended into a lampbrush configuration and are very active in RNA synthesis (Gilbert and Raunio, 1997). These specialized chromosomes direct the metabolic activities of the developing cell. Along with the lampbrush chromosomes, gene amplification also occurs during this stage (Gilbert and Raunio, 1997). During gene amplification ribosomal RNA (rRNA) is replicated for the synthesis of ribosomes, which are necessary for synthesizing proteins. Proteins are needed to supply the growing oocyte as well as carry the embryo through the first stages of development.

Diplotene is also a period of oocyte growth and differentiation. The oocytes grow mainly by accumulating yolk through vitellogenesis. Vitellogenin may be an important route of exposure to toxicants, functioning as a carrier protein for inorganic phosphates, lipids, carbohydrates, and metals that ultimately get incorporated into the oocyte (Ghosh and Thomas, 1995). Once incorporated into the oocyte, vitellogenin is converted into yolk proteins (Wallace and Bergink, 1974). A majority of the yolk accumulates in the vegetal hemisphere of the growing oocyte. This hemisphere is yellowish in color and contains mRNAs essential for organism development. The other half of the oocyte, the animal hemisphere, is darker in pigmentation and contains the nucleus, or germinal vesicle. During development, most of the organs come from the animal hemisphere

(Gilbert and Raunio, 1997). The end product of oogenesis, an oocyte, is a highly polarized cell. This cell contains enough material to form a complete embryo in the absence of sperm (Gilbert and Raunio, 1997). Additionally, the oocyte contains numerous morphological and physiological adaptations that permit rapid and controlled embryonic development.

Webb and Smith (1977) examined the accumulation of mitochondrial DNA (mtDNA) during oogenesis and the effects upon embryos. As the oocyte grows, mitochondria accumulate in the germinal vesicle forming the mitochondrial cloud or Balbiani body (Gilbert and Raunio, 1997). Mitochondria are the sites of oxidative phosphorylation reactions that result in the formation of adenosine triphosphate (ATP) (King and Stansfield, 1990). Therefore, the interruption of the accumulation of mitochondria by growing oocytes has a direct effect upon the energy conversion ability of the embryo. The number of mitochondria present in a fully-grown oocyte is the sole source of mitochondria for the growing embryo (Webb and Smith, 1977).

Oocytes are also involved in protein incorporation. Wallace et al. (1970) found that developing oocytes from hCG-stimulated females sequestered proteins at a higher rate than oocytes from non-stimulated females. Oogenesis is also a period of great histone synthesis (Woodland and Adamson, 1977). Histones are proteins about which DNA winds (Gilbert, 1994). They function in the coiling of DNA to form nucleosomes, which are the basic sub-unit of eukaryotic chromosomes. Unfertilized eggs contain a pool of histones, enough to support nuclear replication through cleavage (Woodland and Adamson, 1977).

Oocyte maturation begins when LH stimulates the follicle cells around the oocyte to produce progesterone (Gilbert and Raunio, 1997). Upon oocyte maturation, meiosis resumes and the germinal vesicle breaks down. During GVBD microvilli retract, nucleoli

disintegrate and the lampbrush chromosomes contract. The oocyte divides and becomes arrested for a second time in metaphase II. The course of *Xenopus* oocyte maturation and the associated biochemical changes have been extensively researched (Sato and Koide, 1984; Cicirelli and Smith, 1985; Taylor and Smith, 1987). Cicirelli and Smith (1985) found that levels of cyclic adenosine monophosphate (cAMP) declined upon oocyte maturation. Exposure to toxicants that interfere with the decline in cAMP levels cause the oocyte to remain in meiotic arrest thereby impeding maturation. Fertilization is the next signal that allows completion of meiosis and the subsequent fusion of pronuclei. The new embryo utilizes its store of histones, energy, mRNA, and proteins to proceed rapidly through development.

The wealth of information regarding the biology, physiology and development of *Xenopus* makes it an excellent study animal (Deuchar, 1975; Muller, 1997). It is easily maintained and its asynchronous oogenesis and breeding reliability allow for year-round reproductive experimentation.

D. The toxic effects of cadmium

Cadmium is a biologically non-essential heavy metal (Eisler, 1985; Webster, 1990), and is a known teratogen and carcinogen. It is used in electroplating, pigment production, and manufacture of plastic stabilizers and batteries (Eisler, 1985). Human exposure to Cd occurs through a variety of sources including smelter fumes and dust, incineration of cadmium-bearing materials and fossil fuels, municipal wastewater and sludge discharge, cigarettes, and some food items (Eisler, 1985). Cadmium accumulates in vertebrates through the food chain and is eliminated very slowly (Vogiatzis and Loumbourdis, 1997). It is essentially absent from the human body at birth, but is readily

taken up, so by age 50 an adult may have between 5 and 20 mg of Cd present in their body depending upon lifestyle and where the person resides (Kostial, 1986).

The TI for $CdCl_2$ was computed from FETAX data by Sabourin et al. (1985) and by Sunderman et al. (1991). Sabourin et al. (1985) calculated a TI of 1.3 for $CdCl_2$. Sunderman et al. (1991), however, calculated the TI of $CdCl_2$ to be 8.6 therefore labeling $CdCl_2$ as a strong teratogen. The differences between the results of the two studies are due to differences in experimental methodology. Sabourin et al. (1985) tested the $CdCl_2$ using an early version of FETAX that called for the embryos to be kept at a colder temperature then the later, ASTM (1992) approved procedure. In addition, the results were based on a single assay and the test compound was dissolved not in the FETAX medium but in water purified by reverse osmosis and then adjusted to the proper hardness with calcium carbonate (Sunderman et al., 1991).

Cadmium toxicity to humans as well as other animals has been extensively examined. Researchers using a variety of mammalian models have examined the toxicity of Cd to adults and fetuses (Lohiya, 1976; Watanabe et al., 1977; Francis ,1984; Soukupova and Dostal, 1991; De et al., 1993; Bokori et al., 1996; Hamada et al., 1998). Rats that were exposed to Cd for eight weeks experienced an increase in spleen weight and an increase in the amount of damaged red blood cells which led to a cadmium-induced anemia (Hamada et al., 1998).

Eisenmann and Miller (1994) found that although Cd is transferred across the human placenta, uptake by the fetus is limited. Exposure to Cd does, however, produce adverse effects on the production and release of hCG and LH (Eisenmann and Miller 1994, Paksy et al., 1997). In mammals, hCG is responsible for causing placental and maternal ovary cells to produce progesterone. Progesterone is a steroid hormone that keeps the uterine wall full of blood vessels (Gilbert, 1994). Cadmium has been shown to

diminish progesterone production in human ovarian granulosa cells (Paksy et al., 1997). Thus, Cd exposure can cause the destruction of the placenta during the last three months of pregnancy (Kostial, 1986). Cadmium also decreases the level of preovulatory LH in the blood and inhibits ovulation (Paksy et al., 1997).

Cadmium affects the reproductive organs of both males and females. A single Cd injection caused reversible destruction of the testes of male squirrels, while repeated injections caused a significant decrease in testicular weight, seminiferous tubule diameter and a decrease in androgen production (Lohiya, 1976). In female mice, it was shown that Cd caused hemorrhages in the ovaries (Watanabe et al., 1977), delayed embryo implantation or caused a complete pregnancy failure (De et al., 1993).

Oogenesis is also affected by cadmium. Adult female mice exposed to cadmium had a decreased number of oocytes in the ovaries, ovulated fewer oocytes and showed an increase in degenerated oocytes (Watanabe et al., 1977). Additionally, there were numerous chromosomal anomalies in the ovulated oocytes such as hypohaploidy, hyperhaploidy and diploidy. Aneuploidy is a condition in which the cell or an organism possesses fewer or more chromosomes than the exact haploid number (Mailhes et al., 1986). Aneuploidy in humans arises primarily in the germ cells of the female. A review of 72 papers by Mailhes et al. (1986) on aneuploidy in mammalian female germ cells revealed that cadmium chloride ($CdCl_2$) caused either hyper- or hypodiploidy in oocytes during metaphase II.

A significant portion of work in the field of Cd toxicity has been conducted with non-mammalian vertebrates. Broiler chickens exposed to Cd for prolonged periods developed subacute-chronic tissue changes in the kidneys and the mass of their hearts and livers significantly increased (Bokori et al., 1996). A significant loss in testicular mass was also reported among exposed male chickens (Bokori et al., 1996). When female

laying hens were exposed to drinking water contaminated with Cd, egg production, egg weight and hatchability decreased while embryo mortality significantly increased (Vodel et al., 1997).

Xenopus laevis, other amphibians, and fish have been used extensively to investigate the adult, reproductive and embryonic toxicity of Cd (Francis et al., 1984; Perez-Coll et al., 1985; Woodall et al., 1988; Sunderman et al., 1991; Sunderman et al., 1995a; Herkovits et al., 1997). The majority of Cd accumulates in the liver, kidney and spleen (Canton and Slooff, 1982; Woodall and MacLean, 1992; Thomas, 1993); however, some amount does accumulate in the ovary and enter the eggs (Papathanassiou, 1986; Pramoda and Saidapur, 1986; Povlsen, 1990; Sunderman et al., 1995b). Sunderman et al. (1995b) proposed that Cd enters the oocytes of *Xenopus* by binding to vitellogenin. Cadmium also exhibits effects on the reproductive endocrine system and on gonadotropin secretion (Thomas, 1993). Gonadal steroid titers were elevated in fish exposed to Cd with subsequent increases in ovarian growth, plasma vitellogenin (VTG), number and hepatic estrogen receptor number (Thomas, 1990). Olsson et al. (1995) found that fish treated with estradiol and then exposed to Cd had decreased levels of plasma VTG. Changes in plasma VTG levels could affect the amount of VTG available to the oocytes and ovarian growth.

Maternal transfer of contaminants to progeny has numerous biological implications. Miller and Amrhein (1995) have documented maternal transfer of organochlorine compounds in a species of lake trout in Lake Superior. They found a positive correlation between the concentration of organochlorines in the muscle tissue of gravid fish and their eggs. Additionally, maternal transfer of Chlordane, a pesticide, has been shown to occur in mayflies (Standley et al., 1994). Cadmium may also be transferred to progeny via maternal exposure. In a field study, Grillitsch and Chovanec

(1995) found high concentrations of Cd and other metals in the spawn of three species of amphibians that they attributed to maternal transfer. They did not perform any laboratory studies to substantiate this claim.

Cadmium can also affect progeny directly. Fertilized eggs from teleosts were exposed to Cd and then allowed to develop (Michibata, 1981; Michibata et al., 1987). The majority of Cd was accumulated in the chorion, embryonic body and yolk sac. As development progressed, the sensitivity of the embryos to Cd decreased. Cadmium also affected the rate of maturation of adult trout thereby delaying oogenesis (Brown et al., 1994) and the rate of oocyte maturation in sea stars (Den Besten et al., 1989). *Xenopus laevis* and *Bufo arenarum* embryos exposed to varying levels of Cd showed an increase in malformation rate and mortality (Perez-Coll et al., 1985; Sunderman et al., 1991; Sunderman et al., 1995a). Malformations exhibited a concentration-dependent pattern and included gut rotation, bent notochord, facial dysplasia, cardiac deformities and dermal blisters (Sunderman et al., 1991).

CONCLUSIONS

Xenopus laevis is an excellent model for examining the possibilities of maternal transfer of Cd to progeny and the effect Cd has upon gametogenesis. The ovary of female *Xenopus* contains several thousand oocytes at various stages of development. The oocytes are easily isolated from the lobe and can be readily staged using an ocular micrometer and a dissection microscope for analysis of a variety of endpoints such as Cd concentration/stage and population of oocytes/stage. Because a single mating can produce thousands of eggs, it is possible to examine Cd concentration at different developmental stages. Larvae of *Xenopus* are transparent allowing for easy observation of

internal malformations. Additionally, numerous developmental studies have been performed with *Xenopus* employing many assays such as FETAX. The use of FETAX aids in determining the effect of Cd on progeny by examining developmental endpoints.

Gametogenesis is a very sensitive and complex stage in an animal's life cycle. Effects of toxicants at this stage can have profound effects later in development. By combining the knowledge gained from multiple toxicity tests covering the various sensitive points of an organism's life cycle, we can better assess the hazards to an animal.

The toxicity of Cd has been well established through experimentation with mammals and non-mammalian vertebrates. Reproductive studies with male and female mammals indicate that Cd has a negative affect on spermatogenesis and on various stages of pregnancy. Although Cd is embryotoxic, teratogenic and fetolethal effects of Cd to mice and rats are only produced by acute maternal exposures to high doses (Webster, 1990). This is due to the placenta-fetal barrier, however the placenta does concentrate Cd and consequently hormone production is altered (Kamrin et al., 1994). In oviparous vertebrates such as amphibians where no such barriers exist between the adult and gamete, acute maternal exposure to Cd may affect the oocyte and this in turn could have profound effects on the progeny.

There are still many gaps in our knowledge of female reproductive toxicology, some of which can be filled by examining the oocytes and the stages of oogenesis. The experimental procedures described in Chapter II of this thesis can be thought of as the first tier of experiments in developing a reproductive toxicity assay. In these experiments, I examined the changes in *Xenopus* oogenesis with exposure to cadmium, thus showing the stages of oogenesis as being a valuable endpoint in assessing toxicity. The information garnered from examining oogenesis can then be compiled with data from developmental

and other toxicity tests to present a more complete picture of reproductive toxicity to an organism.

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Chapter II

THE EFFECT OF SUBUTANEOUSLY INJECTED CADMIUM ON OOGENESIS IN *Xenopus laevis*: APPLICATIONS FOR THE DEVELOPMENT OF A REPRODUCTIVE TOXICITY ASSAY

Introduction

Reproductive toxicity tests have centered mainly on post-fertilization events in mammals and on the male reproductive system. Significant toxicity may be being overlooked by not examining the gametes and gametogenesis of both sexes. At present, a validated reproductive toxicity assay focusing on amphibian gametogenesis has not been developed. However, in order to achieve a thorough assessment of the hazards to an organism, toxicity testing must take into account the different parts of an organism's life cycle. Because full life cycle studies are not practical in most vertebrates it is important to assess toxicity at the stages in an animal's life when sensitivity to toxicants occur (Mattison and Thomford, 1989).

Cadmium (Cd) is a biologically non-essential heavy metal that occurs naturally but is also released into the environment anthropogenically (Eisler, 1985; Webster, 1990; Vogiatzis and Loumbourdis, 1997). It has been shown to accumulate in vertebrates, possibly over a period of years, being eliminated very slowly by the kidneys (Vogiatzis and Loumbourdis, 1997). Cadmium is a known teratogen and carcinogen (Eisler, 1985; Webster, 1990). Researchers using a variety of mammalian models have examined the reproductive toxicity of Cd to adults and fetuses (Loyhiya, 1976; Francis, 1984; Soukopova and Dostal, 1991; Hamada, et al., 1998). Reproductive tissues such as the gonads and uterus readily incorporate Cd (Kostial, 1986) and it is accumulated by the

human placenta. Fetal uptake *in utero*; however, is limited (Eisenmann and Miller, 1994). Acute effects of Cd include testicular necrosis, ovarian hemorrhaging, and delayed embryo implantation (Lohiya, 1976; Watanabe et al., 1977; De et al., 1993). An examination of oogenesis in mice reported an increase in chromosomal anomalies in the ovulated oocytes (Watanabe et al., 1977). Additionally, exposed mice ovulated fewer oocytes and showed an increase in degenerated oocytes (Watanabe et al., 1977).

Fish and amphibians have also been used to investigate the adult, reproductive and embryonic toxicity of Cd. Reproductive endocrine function was altered in adult female Atlantic Croaker (*Micropogonias undulatus*) after Cd exposure (Thomas, 1990) and Cd affected vitellogenin metabolism in flounder (*Platichthys flesus* L.) (Povlsen et al., 1990). Uptake, distribution, and stage sensitivity of Cd to the eggs of the Medaka (*Oryzias latipes*) has been examined (Michibata, 1981; Michibata et al., 1987). The earliest stages of development were the most sensitive and fertilized eggs of the Medaka accumulated most of the Cd in the chorion. Brown et al. (1994) exposed Rainbow trout (*Onchorynkiss mykiss*) and Brown trout (*Salmo trutta* L.) to Cd for periods of up to 90 days. Although continuous exposure did not affect the survival and growth of the adults, eggs from *O. mykiss* failed to develop to the fry stage at 1.8 and 3.0 µg/l while oogenesis in *S. trutta* (L.) was delayed at 9.3 and 29.1 µg/l.

Amphibians represent an ideal model of vertebrate development (Muller, 1997). The study of *Xenopus* oogenesis has contributed to our understanding of human oogenesis. Dumont (1989) developed the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) as a developmental toxicity test. It was standardized by Bantle et al. (1989) and is currently widely used to screen for teratogenic risks from chemical agents (ASTM, 1992). Cadmium was found to cause developmental malformations at numerous concentrations in studies of embryotoxicity using FETAX (Sabourin et al., 1985;

Sunderman, et al., 1991; Sunderman et al., 1995a; Sunderman et al., 1995 b; Kotyzova and Sunderman, 1998). However, the effect of Cd on oogenesis in *Xenopus* or other amphibians, specifically the changes in oocyte population at each stage of oogenesis, has not been well studied. Pramoda and Saidapur (1986) exposed the Indian bullfrog (*Rana tigerina*) to cadmium chloride ($CdCl_2$) and then examined the ovaries and oviducts. They found that the weight of ovaries and oviducts of $CdCl_2$ exposed frogs was decreased and that the vitellogenic growth of oocytes was impaired. Their conclusions concerning the growth of the oocytes was based on histological observations only.

This study describes experiments in which adult female *Xenopus* were exposed to Cd via subcutaneous (s.c.) injection for 21 days. The relationship between the percentage of ova at the stages of oogenesis in control frogs and exposed frogs was assessed. In addition, the amount of Cd contained within ovaries and oocytes was analyzed. Furthermore, treated females were mated to untreated males and the effect of Cd on progeny (F1 generation) in relation to Cd concentration within the ovary examined. The results of this study evaluate endpoints for a standardized reproductive toxicity assay currently being developed by our laboratory.

At the onset of this study female *Xenopus* were exposed to Cd immediately following the acclimation period. These animals did not receive human chorionic gonadotropin (hCG) prior to Cd exposure and are referred to as non-stimulated. Dumont (1972) suggested that oocytes from non-stimulated frogs were not as metabolically active as those from females stimulated with hCG. When hCG is administered to female *Xenopus*, they ovulate most of their mature oocytes and begin a period of vitellogenesis (Dumont, 1972). Therefore, hCG promotes an increase in protein incorporation (Wallace et al., 1970). Keem et al. (1979) measured the growth rate of oocytes in laboratory-maintained *Xenopus* and noted that even when all environmental conditions were

controlled the patterns of oocyte growth between non-stimulated and hCG-stimulated frogs were highly variable. Oocytes from non-stimulated frogs progressed from stage III to stage VI in 16-24 weeks, while the time period for the same growth in hCG-stimulated frogs was 9-12 weeks. In order to standardize reproductive status, decrease variability, and to achieve the greatest uptake of Cd by the ovaries while keeping the length of the toxicity assay as short as possible, we used hCG-stimulated frogs for most of the present study. The greatest amount of oocyte growth occurred 20 to 42 days after hCG-stimulation (Keem et al., 1979). To target this critical oocyte growth phase, exposure to Cd began 20 days after hCG-stimulation and the frogs were exposed for 21 days.

Route of exposure influences toxicokinetic properties and resultant toxicity (Delistraty et al., 1998). Subcutaneous injection via the dorsal lymph sac was chosen as the route of exposure in the present study. The emphasis was not to emulate natural exposure conditions and assess risk, but to ascertain the hazards of cadmium on the stages of oocyte development. Although oral exposure is one of the most natural means of exposure it is not easy to assess the amount of toxicant the animal receives and the toxicant may affect the animal's appetite (Bantle, unpublished data). Fort et al. (unpublished data) have shown that Cd injected s.c. is readily taken up by the ovary in amounts higher than dermal or oral exposure or interperitoneal (i.p.) injection.

Materials and Methods

Frog Maintenance and Culture

Mature female and male *Xenopus* were obtained from Xenopus I (Dexter, MI). The animals were allowed to acclimate for a period of 14 days before being number branded with liquid nitrogen for identification. Each experiment was composed of one

control group and two treatment groups with each group containing the same number of frogs. Frogs were randomly divided into treatment groups and housed in six identical heavy plastic laundry tubs (30.5 cm x 30.5 cm x 53.5 cm) containing 15 L of filtered, dechlorinated water. No more than four frogs were housed per tub. The water was changed three times weekly; temperature was kept at 21° C (± 2°C) and monitored daily. Frogs were housed in a room with a photoperiod of 12h light: 12h dark. They were fed *ad libitum* a diet of ground beef liver and lung containing vitamin supplements (Poly-Vi-Sol, Mead Johnson Co. approximately 0.3 ml/lb) three times per week. Frogs were weighed weekly and behavior was monitored three times per week to assess adult toxicity.

Cd Injection into Frogs

Frogs were injected s.c. into the dorsal lymph sac every other day for 21 days with either 0.7% saline or Cd as $CdCl_2$ (CAS# 10108-64-2) in saline solution at the dose levels (mg/kg body weight) previously determined from rangefinder tests. Frogs in experiment 1 (3.0 or 5.0 mg $CdCl_2$/kg body weight) and experiment 2 (0.5 or 5.0mg/kg) were injected with $CdCl_2$ immediately following the acclimation period (non-stimulated). Frogs in experiments 4 (0.5 and 3.0 mg/kg), 5 and 6 (0.75 and 1.0 mg/kg) were induced to ovulate 20 days prior to beginning Cd exposure by injection with 800 IU hCG (Sigma #CG-5) (hCG-stimulated).

Effect of Cd on Oogenesis

Within 96 hours after the final injection, frogs were anesthetized with MS-222 until unresponsive and then killed by cervical dislocation. Ovaries, livers and spleens were immediately removed and weighed. Necropsies were performed noting any gross lesions or tumors. Livers and spleens were stored in 3% formalin while ovaries were placed in Barth's medium (Smith et al., 1991). A lobe from the ovary was removed immediately and ova were isolated using collagenase according to Smith et al. (1991).

Ova were subsequently staged by diameter (using an ocular micrometer) and morphology as per Dumont (1972) and stored in 3% formalin. Ova at stages II - VI of oogenesis were compared between control frogs and treated frogs. As per Dumont (1972), stage I oocytes were not examined as it was difficult to get an accurate count due to their small size and transparent appearance. The organ weight to body weight ratio for ovaries, livers, and spleens was calculated and the data analyzed for non-stimulated and stimulated frogs.

Effect of Cd on Progeny

Treated and control females were mated to untreated males (experiments 4-6). Animals were induced to breed by injection with hCG (800 IU - females, 400 IU - males). Mating behavior was observed and deviations from normal recorded. Following successful mating, 200 eggs from each pair were double sorted according to standard ASTM FETAX procedures (Bantle et al., 1991) and cultured for 96 h in an incubator at 24° C. FETAX solution was changed every 24 h in all dishes except dishes containing embryos to be used for Cd content analysis (ICP-MS analysis). After 4 days (Stage 46), mortality of the embryos was assessed, embryos were anesthetized in 4% MS-222 and fixed in 3% formalin. Embryos were scored for malformations according to the FETAX protocol and the head-to-tail length was measured.

Determination of Cd Concentration

For analysis of Cd in ovaries, eggs and embryos (experiments 2-6), samples were homogenized and acidified with trace-metal grade nitric acid (HNO_3) to pH 2. Embryo samples for Cd content were taken at 24, 48, 72 and 96 h post fertilization from exposed and control females. Samples and blanks were sent to the U.S. Army Center for Environmental Health Research (USACEHR) (Ft. Detrick, MD) for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis. Techniques for sample processing and microwave digestion were conducted to maximize recovery and to minimize extraneous

metal contamination using Environmental Protection Agency Method 6020. Once the samples arrived at Ft. Detrick they were transferred to acid washed Teflon digestion vessels. Additional HNO_3 was added to make the final volume 10 ml. Samples were digested by microwave using the CEM Microwave Sample Preparation System (CEM Corp., Matthew, NC). Following digestion, the samples were diluted to 40 ml with distilled water and filtered through a 0.45 μm Teflon membrane. Cadmium standards were prepared at 0, 1, 10 and 100 μg/L. An internal standard of Yttrium 89 at 1 ppm was added prior to nebulization. Following standardization the calibration curve was verified by analyzing a 100 μg/L calibration standard and a blank. The check standard was repeated at a 10% frequency during the analysis and after the analysis was completed. The acceptable range for the check standard was +/- 10% of the actual value.

Analyses were performed on all samples using a Hewlett Packard Model 4500 Inductively Coupled Plasma Mass Spectrometer. Final Cd concentrations were corrected for internal standard recovery, analysis dilution, digestion volume and the original mass of the sample. The lower limit of sensitivity was 0.1 μg/g wet weight of the samples. Spiked sample recovery percentages were 55.8% (experiment 2), 86.5% (experiments 4-5), 102.9% (experiment 6).

Statistical Analysis

All data were initially tested for normality and homogeneity of variance. Data were unable to be transformed due to numerous zero values. Oogenesis data were not normally distributed, therefore to determine differences in the stages of oogenesis between control and exposed frogs a Kruskal-Wallis One Way ANOVA on Ranks and Dunn's Test for comparison against the control were performed. Organ-to-body weight ratios between control and treated frogs were normally distributed and a one-way ANOVA and Dunnett's Test were performed (Zar, 1984). The data were initially

analyzed using a two-way ANOVA to evaluate time of experiment and exposure effects (Zar, 1984). Analysis of embryo mortality and malformation rates was performed using the Chi-Square test of equal proportions and Bonferroni confidence interval procedure (Byers and Steinhorst, 1984). The Student's t-test was used to examine the differences in embryo length between the exposure groups (Zar, 1984). The data from the 0.5 mg/kg exposure groups was deleted for mortality, malformation, and length analysis due to the small sample size (n = 1).

Concentrations for ICP-MS were reported as μg/g wet weight. Concentrations below the detection limits of the ICP-MS were represented as zero for statistical analysis. Data were not normal and unable to be transformed therefore a Kruskal-Wallis and Dunn's test of multiple comparisons were employed to determine differences between Cd concentration in the ovaries of control and treated animals (Zar, 1984).

All statistical analyses were performed at the $p < 0.05$ level of significance. ANOVAs, Kruskal-Wallis Tests and multiple comparison procedures were performed with the SigmaStat software from SPSS Science, Chicago, IL. The Student's t-test, Chi-square test, and Bonferroni procedure were performed using the Statistical Analysis Software from SAS Institute, Cary, NC.

Results

Oogenesis

Non-stimulated Frogs

Cadmium had a significant effect on oogenesis in non-stimulated frogs (Figures 1 and 2). In non-stimulated frogs there was a significant decrease in the percentage of stage II oocytes when compared with controls at 3.0 and 5.0 mg/kg ($p < 0.0001$). There was a decrease, relative to controls, in stage III oocytes from frogs exposed at 5.0 mg/kg ($p < 0.0001$). All exposure groups exhibited a decrease in the percentage of stage IV oocytes ($p < 0.0001$) relative to controls. Compared with control frogs, the percentage of stage V oocytes significantly increased at 3.0 and 5.0 mg/kg ($p = 0.0013$). The percentage of stage VI oocytes in all exposure groups was different from controls ($p < 0.0001$). Furthermore, there was a significant increase in atretic eggs at 3.0 and 5.0 mg/kg ($p < 0.0001$) when compared with control frogs.

Estimation of the total number of oocytes/g ovary at each stage (Figure 2) revealed the same trends and statistical differences as in the percentage data (Figure 1) for stages II, III, IV and atretic oocytes. Statistical examination of the total number of oocytes revealed no significant difference in the number of stage V oocytes at all exposures while the number of stage VI oocytes decreased in the 3.0 and 5.0 mg/kg exposure groups ($p < 0.0001$).

Stimulated Frogs

Cadmium also affected oogenesis in hCG-stimulated frogs (Figures 3 and 4). There was a decrease in the percentage of stage II oocytes, relative to controls, in the 3.0 mg/kg exposure group ($p = 0.04$) (Figure 3). The percentage of stage III oocytes was also significantly decreased at 3.0 mg/kg ($p = 0.007$) compared with controls. There was a

significant difference in the percentage of stage IV oocytes between the exposure groups ($p < 0.001$) but not between control and exposed frogs. The percentage of stage V oocytes at 0.5 mg and 3.0 mg/kg decreased ($p = 0.03$) relative to control frogs. Statistical analysis of the stage VI oocytes revealed a significant difference ($p = 0.006$), however because three exposure groups had a median value of 0.00, a multiple comparison test could not be used to make comparisons between the control and exposure groups. When compared with controls, there was a significant increase in the percentage of atretic oocytes in all exposure groups ($p < 0.0001$). Oocytes from Cd-exposed frogs exhibited severe morphological changes from control, frogs (Figure 6). Numerous oocytes from 0.5 mg/kg exposed frogs displayed a speckled or mottled appearance. Completely degenerated oocyte follicles were most common in frogs exposed at 3.0 mg/kg.

Estimation of the total number of oocytes/g ovary revealed basically the same trends in all stages across all exposure groups (Figure 4). There was a significant decrease compared with control frogs in stage II oocytes at 3.0 mg/kg ($p = 0.04$). In stage IV oocytes, there was a significant difference from controls between the 1.0 mg/kg and 0.5 and 3.0 mg/kg exposure groups only ($p = 0.0001$). There was a decrease in stage VI oocytes ($p = 0.03$) between exposure groups, however due to the high incidence of zeros in the data set it was not possible to run a multiple comparison test against control frogs. All exposure groups exhibited a significant increase in atretic oocytes ($p = 0.0004$) relative to controls.

Cd effect on progeny

In four experiments, 12 out of 22 (54.5%) control animals mated successfully, 1/9 (11%) at 0.5 mg/kg, 4/14 (28.6%) at 0.75 mg and 1.0 mg/kg, and 0/8 (0%) animals mated successfully at 3.0 mg/kg. The results of the modified FETAX test on the progeny from females that did mate successfully are shown in Table 1. Because the percentage of

successful matings was extremely low at 0.5 mg/kg (1 out of 9) and zero at 3.0 mg/kg statistical analysis of the mortality, malformations, and growth was limited to the control, 0.75 mg, and 1.0 mg/kg groups. Mortality and malformations appeared to decrease with increasing Cd concentration. The Chi-square test indicated that the probability of mortality and malformations is dose-dependent ($\chi^2 = 252.1289$, $p < 0.001$ and $\chi^2 = 44.4026$, $p < 0.001$, respectively). The Bonferroni confidence interval procedure showed that the difference in mortality lies between the control and 0.75 mg/kg exposure group. Statistical analysis of the head-to-tail length data revealed no significant difference between progeny from exposed females and controls ($p = 0.60$).

In malformed progeny from control females, edema was observed most frequently (66.7% of malformed embryos). The most common malformations in embryos from Cd-exposed females, including the 0.5 mg/kg group, are shown in Figure 5. The highest incidence of malformations in the 0.75 and 1.0 mg/kg groups was in the notochord (61.3 % and 61.9%, respectively). Notochord malformations mainly occurred at the proximal end causing the body axis of the embryo to curve either vertically or horizontally (Figure 7). Because the tails were malformed, embryos swam abnormally, i.e. in circles, after 72 h. Embryos with and without notochord deformities from females exposed at 0.75 mg/kg were allowed to grow past 92 h. All embryos with notochord deformities died within 116 h post-fertilization while the non-malformed embryos (n=10) from the same exposure group survived beyond 140 h. Cadmium also increased the incidence of gut malformations. In the 0.75 mg/kg group 38.7% of embryos and 57.1% of embryos from the 1.0 mg/kg group exhibited loosely coiled guts (Figure 8). Eye deformities were also observed which included misshapen lenses and abnormal eye placement. Other malformations observed included misshapen and shortened heads and deformed mouths.

Cd content in ovaries and embryos

ICP-MS analysis was conducted to examine the Cd concentration in frog ovaries and progeny. Table 2 shows the ovary Cd concentrations in non-stimulated and stimulated frogs and also the percentage of Cd injected that was accumulated by the ovary. The Cd concentration in the ovaries of non-stimulated frogs was significantly different from control frogs at the 5.0 mg/kg exposure level ($p = 0.004$). In stimulated frogs, there was a significant increase in Cd concentration when compared with controls at the 0.5 mg and 3.0 mg/kg levels ($p < 0.0001$). Cadmium concentrations within the ovaries ranged from 2.29 to 15.08 μg/g wet. The percentage of Cd injected that was accumulated by the ovary tended to decrease with increasing Cd dosage. Cadmium was readily detected by ICP-MS analysis in progeny of Cd-exposed females (Table 3). Statistical analysis on these preliminary results indicated no significant difference in fertilized egg or embryo Cd concentrations between control and exposed frogs. The data indicated that there was a greater concentration of Cd in embryos at 24 h than in fertilized eggs.

Cd effect on organ weight

Analysis of the ratio of the organ weight to body weight of the liver, spleen and ovary indicated that in non-stimulated frogs there is a significant increase in the spleen-to-body weight ratio in the 0.5 and 3.0 mg/kg groups ($p < 0.0001$) (Table 4). Statistical analysis revealed no differences in the ratios for the liver, spleen, or ovary in stimulated frogs. Frogs exposed at 3.0 and 5.0 mg/kg exhibited a higher incidence of edema exhibited by large quantities of yellowish fluid within the body cavity.

Discussion

Major changes in the progression of oogenesis and in the morphology of oocytes were evident in *Xenopus* exposed to sublethal levels of $CdCl_2$ for 21 days. Additionally, changes in spleen weight in Cd-exposed frogs relative to control frogs was observed. Progeny from Cd-exposed females exhibited a higher incidence of mortality and malformation than progeny from control females indicating that Cd may be maternally transferred to progeny.

Morphological changes in oocytes included speckling, mottling and complete degeneration of oocyte follicles (Figure 6). We used Dumont's (1972) stages of oocyte development as the model for sorting and staging oocytes in the present study. Ovaries from control frogs in the present study contained oocytes at all stages and most oocytes appeared healthy. Stage IV - VI oocytes had distinct animal and vegetal hemispheres and relatively few atretic oocytes were visible in the ovary. There was, however, a significant increase in the population of atretic oocytes in Cd-exposed frogs. A high incidence of small, very darkly colored, spheres present only in the theca were common in oocytes from Cd-exposed frogs. Dumont (1972) categorized these as completely degenerated oocyte follicles containing densely packed pigment. Our findings are consistent with those of Pramoda and Saidapur (1986) in which *Rana tigerina* were exposed to 600 μg $CdCl_2$ via i.p. injection. After 30 days they performed a histological examination of the ovaries. They found that Cd severely affected the vitellogenic growth of oocytes and increased the number of atretic oocytes. They hypothesized that Cd affected vitellogenic growth by compromising the function of the liver, altering metabolism, and perhaps reducing the blood and nutrient supply to the follicles. Although no visibly degenerated stage II oocytes were found in this study, the population of stage II oocytes was decreased

at every concentration (Figures 1-4) and the population of atretic oocytes was significantly increased. This suggests that stage II oocytes either underwent atresia or there was no recruitment from the population of stage I oocytes. It is probable that exposure to Cd decreased the percentage of oocytes from each stage of oogenesis, including stage II, thereby increasing the population of atretic oocytes. Davidson (1976) suggested that the *Xenopus* ovary contains a reserve of stage II oocytes from which groups are selected to undergo continued oogenesis. If this is true, then the decrease in the population of stage II oocytes from Cd-exposed frogs parlays into a decrease in oocytes in later stages and an overall decline in fecundity. This may permanently affect the ability of the female to reproduce.

The effects of Cd on oogenesis are much more apparent in hCG-stimulated frogs than non-stimulated frogs. As discussed by Dumont (1972), it appears that the ovaries of hCG-stimulated frogs in the present study have a higher metabolic activity and thereby are more actively involved in taking up Cd. Such a phenomenon also occurs when trypan blue is injected into hCG-stimulated and non-stimulated frogs (Dumont, 1972). Trypan blue is actively taken up by oocytes in hCG-stimulated frogs; however, the uptake is virtually nonexistent in non-stimulated frogs. The overall metabolic activity of oocytes from non-stimulated frogs is markedly diminished. Therefore, although the morphology of oocytes is the same at the identical stages in non-stimulated and hCG-stimulated frogs, there are cytological and physiological differences (Dumont, 1972).

The interaction of cadmium and vitellogenin synthesis has been the focus of many studies. Sunderman et al. (1995b) have shown that Cd binds to the vitellogenin yolk protein lipovitellin 1 in *Xenopus* and could be a mechanism for reproductive toxicity of Cd following environmental exposure. They hypothesized that Cd absorbed by the female could bind to plasma vitellogenin in place of zinc (Zn), enter oocytes by

endocytosis and become deposited in the yolk platelets. Cadmium, when it is bound to vitellogenin, also becomes incorporated in the oocytes of Atlantic croakers (*Micropogonia undulatus*) (Ghosh and Thomas, 1995). Therefore, the binding of cadmium to vitellogenin could significantly increase the accumulation of the metal. In a preliminary study, our data indicate that Cd is incorporated into stage IV and V *Xenopus* oocytes, and atretic oocytes of Cd-exposed females. We are continuing to gather data on the concentration of Cd in oocytes at various stages.

Povlsen et al. (1990) discovered that one injection of 2 mg Cd/kg body weight caused a significant decrease in the amount of circulating plasma vitellogenin and calcium in the flounder (*Platichthys flesus* L.). Additionally, Cd exposure decreased the ratio of RNA to DNA in the liver. This indicates that Cd may interfere with transcription which, in turn, inhibits the vitellogenin synthesis. Transcriptional down-regulation of vitellogenin synthesis in response to Cd exposure has been reported by Olsson et al (1995) in the rainbow trout (*Oncorhynchus mykiss*). Because oocyte growth in fish and amphibians is largely through the uptake of vitellogenin, the inhibition of vitellogenin synthesis may lead to a decrease in the population of later stage oocytes.

Watanabe et al. (1977) performed chromosome analysis on dd/YF mice. The number of females with abnormal oocytes increased in the Cd-treated groups in conjunction with an increase in the number of oocytes exhibiting chromosome aberrations. They concluded that Cd has the potential to be a mutagen in mammalian meiotic chromosomes. Watanabe et al. (1977) also exposed mice to Cd via a single s.c. injection of $CdCl_2$ and then examined the oocytes and ovaries. They found a significant decrease in the number of oocytes recovered with increasing Cd concentration. We have shown that Cd caused a decrease in the oocyte population at most stages of oogenesis and at most concentrations of Cd tested (Figures 1-4, 6).

Although Cd caused a decrease in the oocyte population in most stages of oogenesis and in most exposure groups, the population of stage V (non-stimulated frogs) and stage IV (stimulated frogs) oocytes at some exposures was greater than in the controls. These findings may be due to the variation in the number of oocytes present in any given stage prior to treatment in captive female *Xenopus* (Dumont, 1972). Physiological changes caused by Cd at stage IV could preclude further oocyte development to later stages. Interestingly, frogs exposed to Cd at a dose of 3.0 mg/kg did not breed (did not go into amplexus) and the percentage of frogs that bred at lower exposure concentrations was very low. Examination of the ovaries of the Cd-exposed frogs revealed that mature stage VI oocytes were present and, presumably, should have been ovulated. Those frogs whose ovaries contained few or no stage VI oocytes did have stage V oocytes. Animals whose ovaries do not contain stage VI oocytes can still be induced to ovulate the most mature (stage V) oocytes upon hCG stimulation (Dumont, 1972). Therefore, Cd exposure may affect physiological or behavioral aspects of ovulation and breeding.

In dd/YF mice, Watanabe et al. (1977) found that the ovary Cd concentration increased relative to the dose. Additionally, the incidence of degenerated oocytes was higher in the treated groups. In the present study the concentration of Cd in the ovaries of Cd-exposed frogs did not increase with dose. The ovaries of the lowest exposure group (0.5 mg/kg) exhibited a higher Cd concentration than the 0.75 and 1.0 mg/kg exposure groups. Additionally, the percentage of Cd that was accumulated by the ovaries decreased with increasing exposure concentration. It is possible that at the concentrations tested the ovary became saturated with Cd causing necrosis of the ovary and the subsequent inability to accumulate any more toxicant. Figure 6 shows the dramatic loss of cells at higher Cd concentrations and illustrates that cell loss may well indicate why less Cd is

present at higher exposure doses. Alternatively, if vitellogenesis is the main mechanism by which the oocytes incorporate Cd and Cd inhibits vitellogenesis, then this may lead to a marked decrease in the uptake of Cd.

The wet weight of the liver, spleen, and ovaries was taken during the necropsy and analyzed in comparison to the body weight (Table 4). We did not find a significant difference in the organ-to-body weight ratios in stimulated frogs. Hamada et al. (1998) found that after eight weeks of Cd exposure, the spleen weight of exposed rats doubled over that of controls. In addition, red blood cell density has been shown to decrease in Cd-exposed rats, causing them to become anemic (Kunimoto and Miura, 1986). These findings are in contrast to data from other species. Jana and Sahana (1988) found that the liver and ovary weights of the freshwater fish *Claria batarachus* (L.) were decreased and the protein content was increased after Cd exposure. Additionally, a significant decrease in ovary weight was noted after a single s.c. injection of Cd into the Indian koel (*Eudynamys scolopacea*) (Sarkar et al., 1976). In the present study, we noted that ovaries from exposed frogs often contained large amounts of fluid in the theca which could produce inaccurate results. Therefore, dry weights will be used in future studies in addition we will analyze the protein content of organs, oocytes, and embryos.

In non-stimulated frogs, we did find a statistically significant increase in the spleen-to-body weight ratio in two exposure groups (0.5 mg/kg and 3.0 mg/kg). It may be possible that because the ovaries in non-stimulated frogs were less metabolically active they sequestered less Cd and the Cd concentrated in the spleen instead causing splenomegaly. Support is lent to this hypothesis when the stimulated frogs are examined. There was no statistically significant increase in the spleen-to-body weight ratio in stimulated frogs. The majority of Cd was probably incorporated into the ovaries instead.

This hypothesis should be tested by examining the Cd concentration in the spleens of non-stimulated and stimulated female frogs.

In addition to examining the effect of Cd upon oogenesis, we were also interested in exploring the possibility of transgenerational effects. Maternal transfer of toxicants to offspring has numerous biological implications such as toxicity to progeny and transgenerational accumulation of contaminants (Standley et al., 1994). Possible mechanisms of maternal transfer in oviparous animals include incorporation into the lipids forming the egg mass, passive diffusion into mature eggs and active transport via vitellogenin. Some toxicants, such as organochlorines, are transferred from female to egg via lipids in mayflies (Standley et al., 1994) and in lake trout (Miller and Amrhein, 1995). Maternal transfer of Cd in mammals and birds is limited. Sato et al. (1996) found that transfer of Cd to eggs of leghorn chickens was restricted even when high amounts of Cd accumulated in the maternal liver. Cadmium accumulated in higher concentrations in the follicle walls rather than the follicle yolks.

Studies examining maternal transfer of toxicants in amphibians have been limited. Grillitsch and Chovanev (1995) performed a field study in which they measured the concentrations of heavy metals and pesticides in anuran spawn, tadpoles, water, and sediment. Spawn, as defined by the authors, are embryos at Gosner (1960) stage 15 ± 2, (neurula). They found Cd, copper (Cu), lead (Pb) and Zn residues in spawn and tadpole samples of *Bufo bufo, Rana dalmatina,* and *Rana ridibunda* at higher levels than sediment concentrations. Although maternal transfer could be a source of metal contamination in spawn, the possibility of water related metal contamination should not be discounted.

We analyzed the Cd content in the fertilized eggs from the mating of control and exposed females to non-exposed males (Table 3). The Cd content was remarkably low, even lower than concentrations found in embryos at 24 h. One possible explanation for

this is the method of weighing the fertilized eggs. Fertilized eggs from exposed females were soft and very fragile and thus, ruptured easily. While determining wet weight, it is probable that egg mass was lost and therefore the Cd content in the fertilized eggs is most likely underestimated. We have since modified our protocol to include dry weight of the eggs and embryos in the determination of Cd content.

We also analyzed the Cd concentration in progeny from Cd-exposed females. We found that embryo mortality from females exposed to 0.75 mg/kg was significantly greater than controls (Table 1). However, embryos from Cd-exposed females contained nearly identical Cd concentrations as embryos from control females (Table 3). It may be possible that the embryos that died contained a higher concentration of Cd than the survivors. Embryos from control females exhibited Cd concentrations that were very similar to the concentrations in embryos from females exposed at the lowest level in a similar study (Kotyzova and Sunderman 1998). However, Kotyzova and Sunderman (1998) exposed female frogs to Cd dermally, stimulated ovulation with hCG, then fertilized the eggs *in vitro*, warranting caution when comparing results. Interestingly, the Cd content in the embryos at 96h was decreased from 24h. In order to determine if the embryos were eliminating Cd over the duration of the FETAX assay, the FETAX solution in the dishes containing the embryos was sampled every 24 h and analyzed for Cd. The Cd concentration of the FETAX solution remained constant, barely above detection limits, throughout the assay. It is possible that the Cd in the solution is too dilute to detect. Therefore, the following warrants further investigation: 1) why is measurable Cd present in the embryos from control females and 2) what is the mechanism for embryonic Cd decline over the course of the FETAX assay?

We found a significant increase in malformations in the F1 generation from females exposed at 0.75 mg/kg. The malformations found in the progeny in the present

study were similar to those found in direct FETAX exposure studies of *Xenopus* embryos to Cd (Sunderman et al., 1991; Herkovits, et al., 1997) and in embryos from Cd-exposed females (Kotyzova and Sunderman , 1998).

A thorough assessment of the hazards to an organism should include toxicity testing encompassing the different stages of an organism's life cycle. Likewise, testing should incorporate all aspects of toxicity including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity. While, full life cycle studies are not practical in most vertebrates due to their long life spans, toxicity can be assessed at the stages in an organism's life cycle when sensitivity to toxicants occurs (Mattison and Thomford, 1989). During reproduction and development, toxicity may occur at numerous stages including gametogenesis, fertilization, and embryogenesis. However, current reproductive and developmental toxicity tests focus heavily on post-fertilization events. Furthermore, reproductive toxicity testing has centered either on mammals, or specifically on toxicant effects upon spermatogenesis. As a result, significant toxicity effects upon oocytes and the stages of oogenesis are understudied. In the present study, we have shown that the stages of *Xenopus* oogenesis are a sensitive and valuable endpoint in reproductive toxicity testing. Likewise, the effect of toxicants on the progeny of exposed females should also be considered.

In summary, we have demonstrated that the stages of oogenesis are sensitive to Cd exposure. Additionally, we have shown that Cd is transferred maternally and that the FETAX assay can be easily modified to demonstrate the effects of maternal exposure to reproductive toxicants upon progeny thereby providing vital information on transgenerational effects. The growth rate of oocytes has been determined and from that the ideal length of exposure and of the assay have been resolved. Furthermore, the use of hCG-stimulated frogs has been shown to produce the consistent results. We have found

the collagenase technique of liberating oocytes in order to sort and stage them to produce accurate results. In order to maintain good statistical power, there should be no less than seven frogs per exposure groups and no less than 200 oocytes should be sorted and staged. Analysis of the Cd content in dry ovaries, spleen, and liver are also informative endpoints.

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APPENDICES

APPENDIX A

TABLES

Table 1. Results of Modified FETAX Toxicity Testing on Progeny of Cd-exposed and control female *Xenopus laevis*. (* indicates $p<0.05$)

** Exposure group (mg/kg)	Mortality (%) & number of frogs	Malformation (%) & number of frogs	Embryo length (mm) mean (sd) & number of embryos
control	8.8 (n=11)	1.63 (n=11)	9.18 (0.036) (n=2044)
0.50	50.0 (n=1)	59.3 (n=1)	8.01 (0.042) (n=15)
0.75	*40.6 (n=4)	*8.91 (n=4)	8.50 (0.072) (n=404)
1.00	13.5 (n=4)	3.52 (n=4)	8.96 (0.083) (n=597)

** frogs exposed at 3.0 mg/kg did not breed

Table 2. Mean (± sd) Cd concentration in the ovaries of non-stimulated and stimulated frogs and the percentage of Cd injected into frog that was accumulated by the ovary. (*indicates $p<0.05$)

	Exposure Group mg/kg	Cd Concentration (μg/g wet weight)	% Cd injected that ovary accum.	n (# frogs)
Non-stimulated frogs	control	0.059 (0.109)	no Cd injected	7
	0.5	0.648 (0.288)	3.107 (1.645)	6
	5	*1.530 (0.292)	0.737 (0.209)	6
Stimulated frogs	control	0.068 (0.057)	no Cd injected	3
	0.5	*9.176 (2.926)	22.21 (7.21)	8
	0.75	5.017 (2.662)	10.08 (5.14)	6
	1.00	6.400 (3.091)	9.13 (3.13)	8
	3.00	*9.230 (3.144)	5.79 (2.17)	14

Table 3. Mean (sd) Cd concentration present in fertilized eggs and embryos of stimulated frogs

Exposure Group mg/kg	Cd. conc in fertilized eggs (μg/g wet weight)	Cd. concentration in embryos (μg/g wet weight) 24 h	48 h	72 h	96 h
control	0.057 (0.035)	0.467 (0.035)	0.365 (0.127)	0.126 (0.035)	0.058 (0.060)
0.75	0.543 (0.670)	0.929 (0)	0.879 (0.103)	1.866 (2.411)	0.089 (0)
1.00	0.505 (0.203)	0.722 (0.417)	1.135 (0.658)	0.551 (0.523)	0.224 (0.243)

Table 4. Mean (sd) organ:body weight ratios in non-stimulated and stimulated frogs. (* indicates $p<0.0001$)

	Exposure Group (mg/kg)	Liver (g)	Spleen (g)	Ovary (g)
Non-stimulated	control	0.042 (0.150)	0.001 (0.001)	0.134 (0.033)
frogs	0.5	0.044 (0.010)	*0.003 (0.001)	0.152 (0.025)
	3.0	0.045 (0.005)	*0.007 (0.015)	0.131 (0.027)
	5.0	0.041 (0.007)	0.001 (0.000)	0.139 (0.030)
Stimulated	control	0.034 (0.005)	0.001 (0.001)	0.105 (0.028)
frogs	0.5	0.109 (0.151)	0.003 (0.002)	0.077 (0.032)
	0.75	0.042 (0.005)	0.001 (0.001)	0.109 (0.055)
	1.0	0.040 (0.008)	0.003 (0.004)	0.143 (0.061)
	3.0	0.0449 (0.013)	0.003 (0.002)	0.086 (0.040)

APPENDIX B

FIGURES

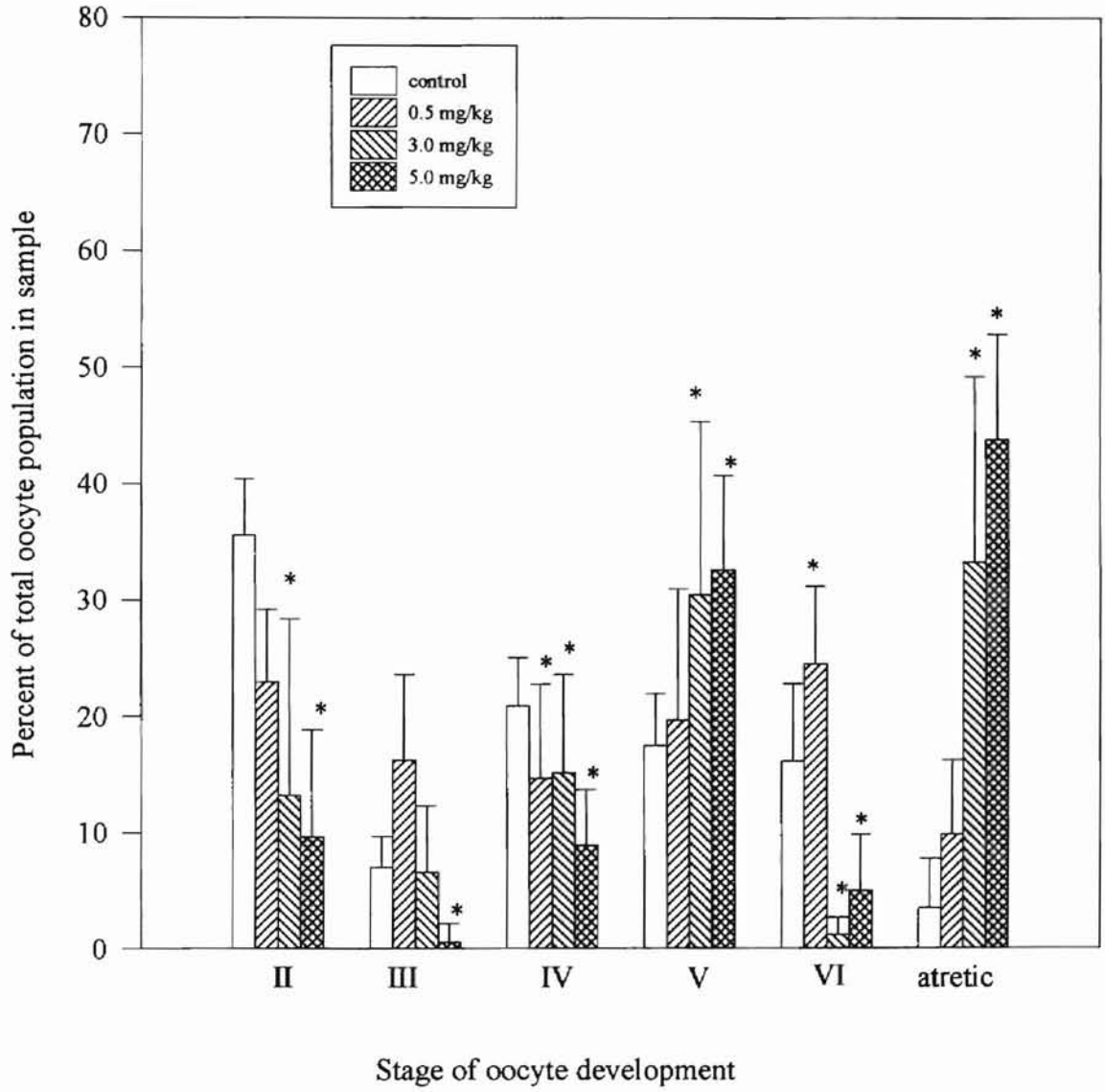

Figure 1. Mean percentage of oocytes present at each stage of oogenesis in non-stimulated frogs. Error bars indicate standard deviation. (* denotes $p<0.05$)

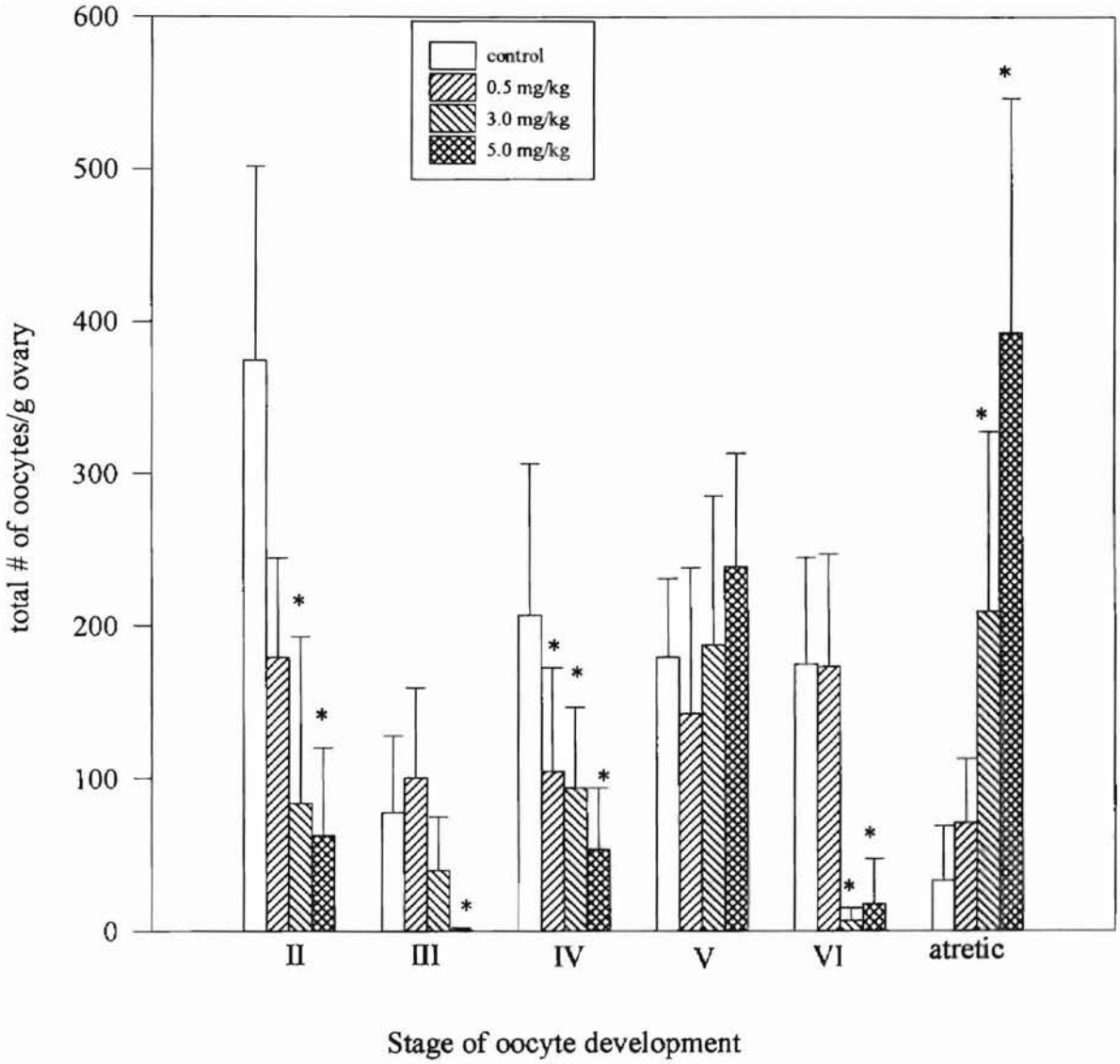

Figure 2. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-non-stimulated frogs. Error bars represent standard deviation. (* denotes $p<0.05$)

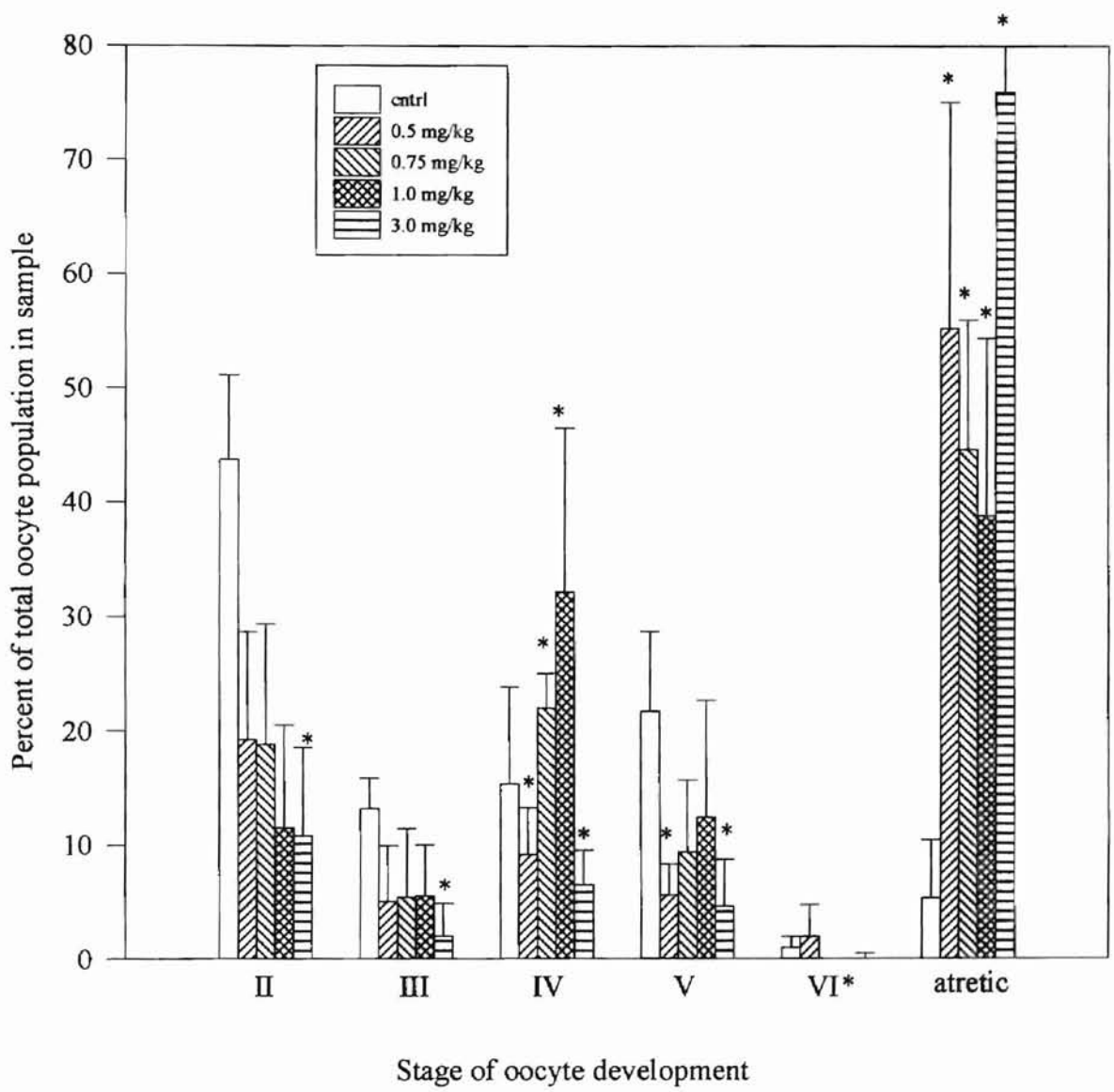

Figure 3. Mean percentage of oocytes present at each stage of oogenesis in hCG-stimulated frogs. Error bars represent standard deviation. (* denotes $p<0.05$)

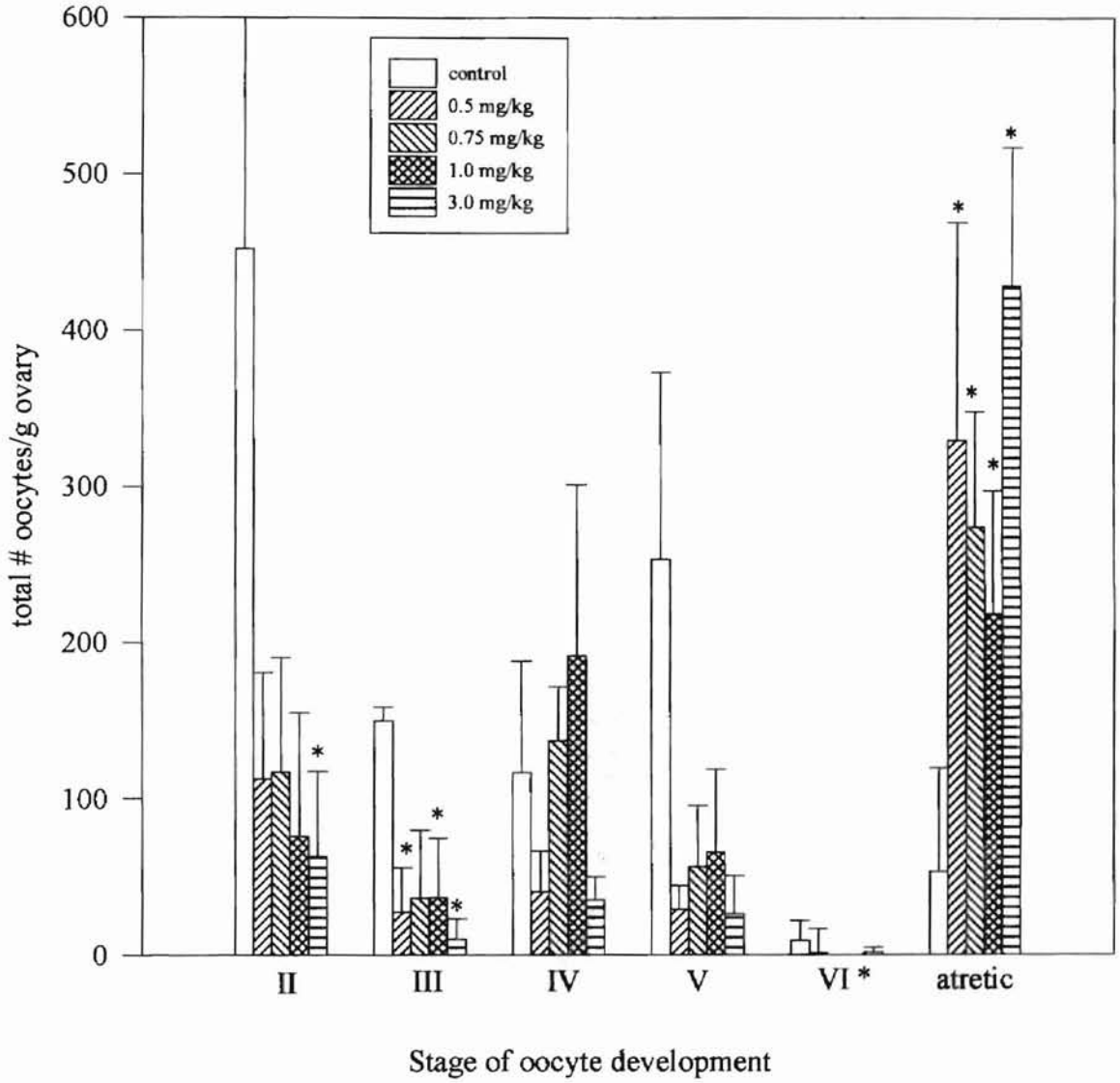

Figure 4. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-stimulated frogs. Errors bars represent standard deviation. (* denotes $p<0.05$)

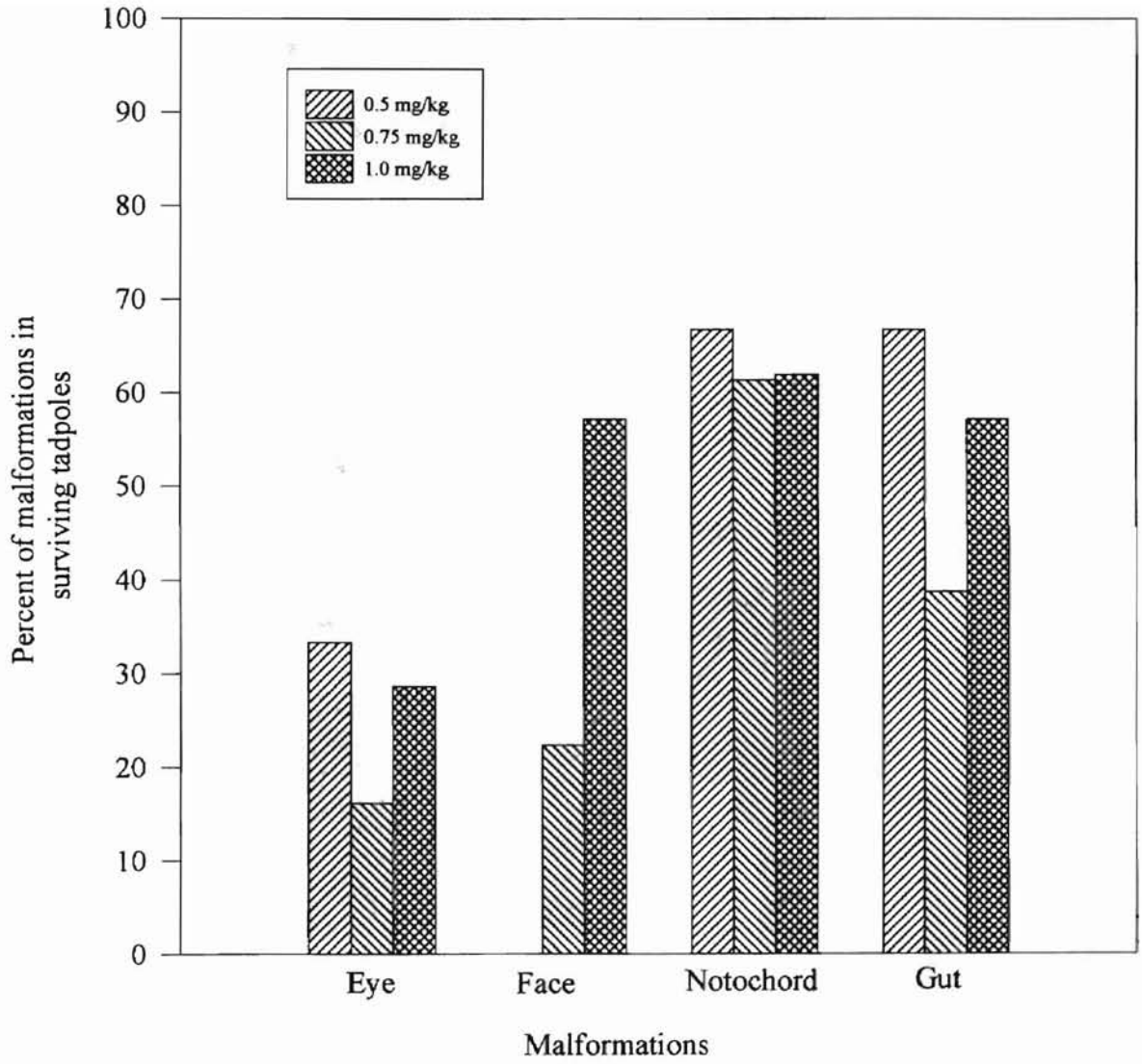

Figure 5. Incidence of malformations in tadpoles after maternal exposure to Cd.

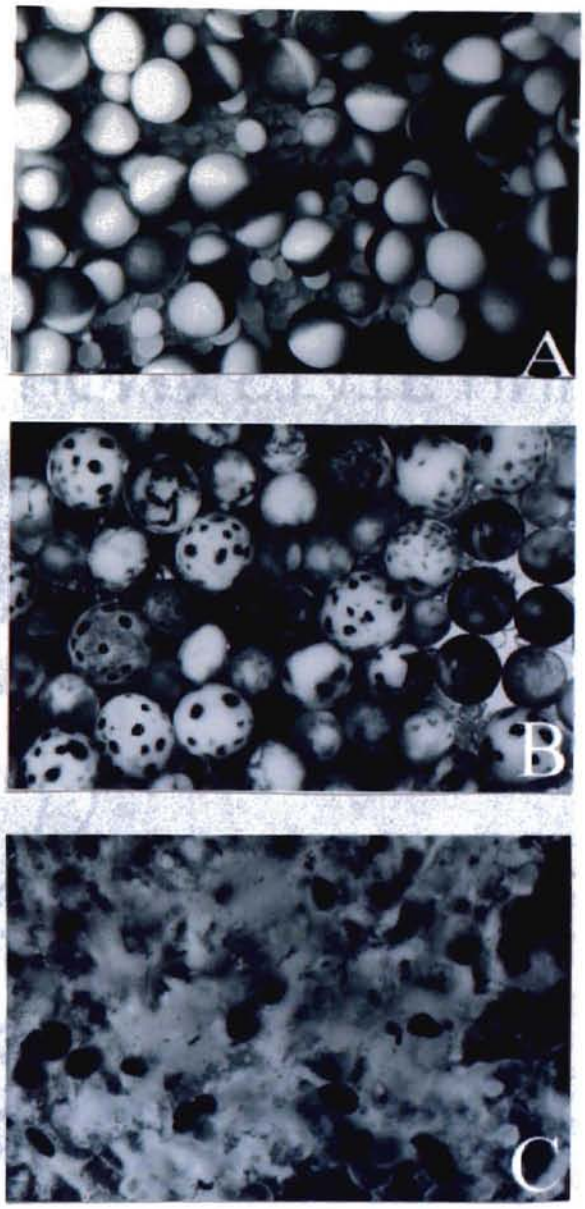

Figure 6. Oocytes from control female (A). Stage II oocytes appear opaque, stage IV-VI oocytes have distinct animal (dark) and vegetal (light) hemisphere. (B) oocytes from female exposed at 0.5 mg/kg. Morphology of oocytes is different from controls. Numerous oocytes are speckled or mottled and viability is questionable. (C) oocytes from female exposed at 3.0 mg/kg. Completely degenerated oocyte follicles appear as darkly pigmented spheres.

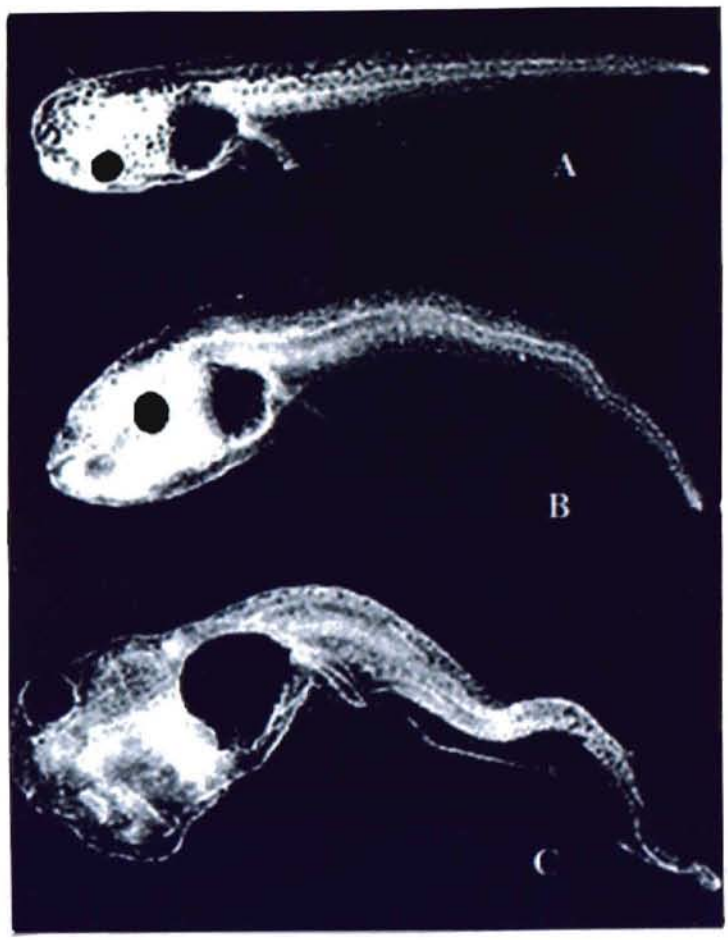

Figure 7. Notochord malformations. (A) embryo from control female. (B) and (C) embryos from female exposed at 0.75 mg/kg.

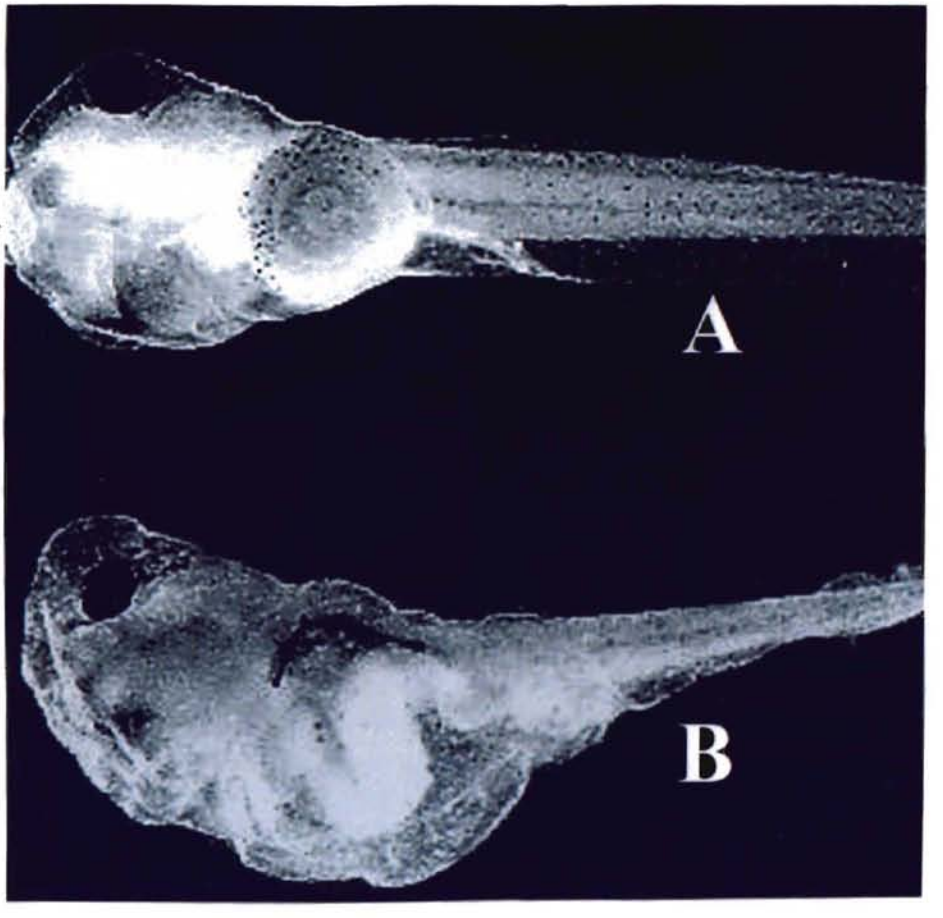

Figure 8. Gut malformations. (A) embryo from control female. (B) embryo from female exposed at 0.75 mg/kg.

VITA

Laila Aida Lienesch

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF SUBCUTANEOUSLY INJECTED CADMIUM ON OOGENESIS IN XENOPUS LAEVIS: APPLICATIONS FOR THE DEVELOPMENT OF A REPRODUCTIVE TOXICITY ASSAY

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