A Study on The Effects of Endocrine Disruptors on Neurodevelopment

By

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Dedications

To the Saint Peter’s University Biology Department

Preface and Acknowledgements

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Abstract

Endocrine disrupting chemicals (EDCs) such as phthalates, bisphenol A, and some herbicides, are exogenous substances with the ability to interfere with crucial signaling chemicals of the endocrine system. Their environmental abundance has been increasing. They have been linked to negatively affect neurodevelopment, reproduction, morphological changes, and other processes. Mechanisms of action of EDCs include estrogen and thyroid hormone disruption. This study was designed to study the effects of the selected EDCs (diethyl phthalates, bisphenol A, amino ethyl propanol, and dimethicone) on neurodevelopment and the morphology of *Xenopus laevis*. Five groups of six *Xenopus laevis* embryos were incubated with the above EDCs, and were compared to controls incubated in dechlorinated tap water. The experimental groups were exposed to $10^{–6}$g/ml of each chemical, which was brought to a 1:100 final concentration with dechlorinated tap water. No significant neurodevelopment differences were observed among the different groups; however, other physical and behavioral differences, including spinal curvatures, swimming patterns, size, pigmentation, and mortality, were significantly altered.
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Introduction:

Endocrine disrupting chemicals (EDCs) are exogenous substances with the ability to cause adverse effects to an organism and/or its progeny by altering functions of its endocrine system (Lintelmann et al., 2003). Many of the EDCs are synthetic chemicals with the ability to interfere with endogenous hormones and
other signaling chemicals of the endocrine system by mimicking their structures and functions. Most commonly found in consumer products, humans are exposed to EDCs through inhalation, ingestion, and dermal contact (Miodovnik, 2011). These chemicals are very abundant in the environment as 87,000 are in use today, and they are a major part of the world’s economy and commerce (Colborn, 2004). Exposure to neurotoxic EDCs during critical periods of development has been suggested to negatively affect sensory, motor, and cognitive function by disrupting neurodevelopment processes (Tilson, 1998). Exploring these suggestions can possibly shed light on the possible involvement of EDCs in developmental disabilities such as autism.

**Introduction to EDCs**

Endocrine disrupting chemicals are most abundant in industrialized areas where contamination is also most probable. Out of the 87,000 chemicals noted as EDCs, approximately 75,500 are industrial chemicals, 3500 are pesticide ingredients, and over 8000 are cosmetics, food additives, and nutritional supplements. The long half-lives of EDCs that makes them beneficial for industrial use are the same properties that make them detrimental to living organisms. Some may not be metabolized, and others may be metabolized into compounds more toxic than the initial compound (Calafat and Needham, 2007; Porte et al., 2006). Despite their widespread use in the environment, EDCs have also been found in internal environments at an alarming rate. A study conducted on the indoor exposure to EDCs detected a presence of 13 to 28 EDCs in air, and 6 to 42
EDCs in dust; all of which were from plasticizers, disinfectants, herbicides, and adhesives. In addition to also detecting some illegal compounds, they found the EDCs present exceeded government guidelines (Rudel et al., 2003).

EDCs can be divided into natural compounds and man-made compounds. Natural compounds, such as phytoestrogens found in infant soy formula, are released into the environment through excretion by various organisms. It was reported by a recent study that the urinary concentration of infants fed soy formula instead of cow’s milk formula was about 500-fold higher in phytoestrogens. Man-made or synthetic compounds are released by point of application, or by volatilization, leakage, and leaching. EDCs include byproducts such as polychlorinated biphenyls, plastics such as bisphenol A, plasticizers such as phthalates, pesticides such as methoxychlor, pharmaceutical agents and herbicides. These chemicals enter the food chain and accumulate in animals higher up in the food chain after exposure by drinking water, contaminated air, ingestion, and contact with contaminated soil (Lintelmann et al., 2003; Diamanti-Kandarakis et al., 2009).

**Mechanisms of Actions of EDCs**

The phenotypic effect of endocrine disrupting chemicals can be both transgenerational and non-transgenerational. In humans specifically, EDCs are stored in tissue such as body fat and they mobilize during maternal egg laying,
pregnancy, and lactation (Colborn, 2003). Many studies have suggested that their effects can be observed at low doses that range in nanomolar and micromolar units of concentration (Vandernberg et al., 2012). Their most common mechanisms of action involve their interference with regulatory pathways at different stages necessary for proper cellular response.

Endocrine disrupting chemicals can act by transactivating classical nuclear receptors, such as those of the thyroid hormone (TH). With their ability to be both agonists and antagonist, EDCs such as plasticizers, herbicides, and food constituents can induce TH gene expression by over 1.5 fold (Hofmann, 2009; de Coster and Van Larebeke, 2012). After binding to receptors and causing conformational change, the receptor itself can act as a transcription factor and interact with cofactors and co-repressors that regulate DNA transcription. Additional genomic alterations employed by EDCs include DNA methylation and interference with the mitotic spindle figure. These alterations can be epigenic as they affect DNA sequence and chromosome segregation that is passed down the germ line.

EDCs can also have nongenomic effects by interfering with membrane bound receptors to initiate a signal transduction similar to endogenous steroid hormones. Their interaction affects the Ca++ influx responsible for intra and extracellular signaling processes, and rapid hormone secretion by inducing exocytosis (Wozniak et al., 2005; de Coster and Van Larebeke, 2012). Accordingly, they have the ability to affect the metabolism of hormones and interfere with
hormonal feedback regulation and neuroendocrine cells. These alterations are most commonly seen with the thyroid hormone due to its high dependence on the neuroendocrine system. Consequently, both genomic and nongenomic mechanisms can be affected simultaneously (De Coster and Van Larebeke, 2012).

**EDCs in Nervous and Endocrine System Disruption**

The neuroendocrine system refers to the physiological combination of the nervous and endocrine system for proper body functioning. In this system, the signals are sent between the brain and glands of the endocrine system, such as the thyroid, that leads to the release of hormones that control important developmental functions (What is The Neuroendocrine System). The thyroid, which is part of the neuroendocrine system, has been suggested as a target for EDCs because the target is the hypothalamic pituitary axis (Gore, 2008).

Many development behaviors depend on the integrity of the hypothalamic-pituitary system, which is vulnerable to neurotoxins that affect cells directly and indirectly (Tilson, 1998). EDCs have the ability to antagonistically mimic hormones, and interfere with hormone-sensitive periods of neural development such as neuronal growth, differentiation, synapse formation, and subsequent behavior (Colborn, 2004). One of the crucial hormones they are known to target is the thyroid hormone, a hormone dependent on the hypothalamus and anterior pituitary for stimulus and production. Many EDCs have a ring system structurally similar to the thyroid hormone (Auf'mkolk et al., 1986).
They can bind to the thyroid receptors and inhibit thyroid receptor-mediated
gene activation (Moriyama et al., 2002). In addition, they have also been found to
interfere with thyrotropin receptors, which disrupt regulation of the thyroid
hormone production (Auf’molk et al., 1985). Because EDCs do not participate in
thyroid hormone-mediated regulation and feedback mechanisms, they negatively
affect the hypothalamic-pituitary-thyroid axis (Schmutlzer et al., 2007).

Poterfield et al. have shown that the development of the nervous system is
highly associated with the presence and circulation of thyroid hormones (1993).
The presence of the thyroid hormone in the cerebellum participates in the
regulation of many genes, including the gene encoding for myelin basic protein
necessary for proper myelination and signal transduction of neurons. Many
studies have also suggested that thyroid hormone directly regulates
neutrotropins, which are involved with neuron growth, neuronal differentiation,
and synaptogenesis (Koibuchi et al., 2000). Accordingly, the neurotoxicity of EDCs
has been reported to induce hypothyroidism, possibly due to interference with
receptors necessary for proper thyroid hormone response. The deficiency of the
thyroid hormone, especially during the critical period of neural differentiation
can cause severe and permanent damages to the anatomy and function of the
nervous system (Bernal et al, 1995). In vertebrates, it imposes a condition known
as cretinism, which results in severe mental retardation and skeletal growth
defects (Hetzel, 1989).
**EDCs and Brain Disorders**

Numerous studies have hypothesized that prenatal exposure to endocrine disruptors may contribute to neurodevelopmental disorders (de Cock et al., 2012). When comparing data on the growth of synthetic chemical production and data on the increasing prevalence of neurodevelopmental disorders such as mood disorders, Autism Spectrum Disorder, and Attention Deficit Disorder, a strong correlation is seen around the 1970s, when the first generation of humans exposed to these synthetic chemicals in the womb began to have their own children (Colborn, 2004). These neurodevelopmental disorders particularly can cause an enormous emotional, mental, and financial toll on individuals due to the compromise of the quality of life and a lifelong disability (Gupta, 2008).

The permeable placenta that links the fetus to the mother enables the passage of the toxic EDCs, as it fails to completely block their transfer from the maternal circulation to the fetus (Fowden et al., 2009; de Cock et al., 2012). In addition, maternal metabolic alterations have been recognized as risk factors to neurodevelopmental disorders. Fetal brain development is highly regulated and influenced by critical metabolic hormones such as the thyroid hormone, which is essential for normal embryonic and fetal neurogenesis (Patel, 1976). Acting antagonistically, EDCs have the ability to bind to the thyroid receptor and inhibit thyroid receptor-mediated gene activation, and cause other alterations as discussed earlier (Moriyama et al., 2002). During the latter half of pregnancy
especially, processes such as myelination and synaptogenesis are particularly sensitive to thyroid hormone alteration (Howdeshell, 1999; Bernal, 1995). In addition, these alterations can inhibit the development of dendrites in cerebellar Purkinje cells, which are very important for motor coordination. Thus, the deficiency of thyroid hormones in utero may be responsible for morphological brain impairment seen in neurodevelopmental disorders, such as autism (Gustavo, 2007).

Objectives

The purpose of this study is to contribute to knowledge concerning the ability of EDCs to induce neurodevelopmental impairment in *Xenopus laevis*, from which a relation may be drawn to humans. Particularly in *Xenopus laevis*, it has been found that thyroxin, secreted by the thyroid hormone, is crucial for metamorphosis and changes in the nervous system during that period. Not only does it affect the expression of 34 genes in the amphibian brain, but it also impacts neural cell proliferation, differentiation, and apoptosis (Denvers et al., 1997; Crump et al., 2002).

The EDCs that were used can be classified into phthalates, herbicides, and BPA. Phthalates are plasticizers that are found in polyvinyl chloride plastics, food packaging, children’s toys, and cosmetic personal care products (Miodovnik, 2011). Phthalates have been shown to exhibit thyroxin T3- antagonistic activity, thus altering neurodevelopment (Sugiyama et al., 2005). Herbicides, such as
atrazine, have been found to cause organ malformations in *Xenopus laevis* only after 6 to 12 hours of exposure as they induce midbrain apoptosis (Lenkowski et al., 2008). BPA, a widely used EDC in dentistry, has been linked to the impairment of neocortical development and changes in the activation of genes that are essential for neuronal differentiation (Nakamura et al., 2007). The human consumption of BPA is approximately 6.6ug/person-day, and it has been found in the concentration of 1-10ng/ml in the serum of pregnant women and the amniotic fluid of their fetus. (Schonfelder et al., 2002; Ikezuki et al., 2002).

The length of the *Xenopus laevis* life cycle is ideal for the observation of neural development in a short time frame. To ensure a wide range observation, *Xenopus laevis* embryos at NF stage 12 (Nieuwkoop and Faber, 1994), the end of gastrulation, were incubated with different EDCs that are most common in the environment in order to determine their effects on neurodevelopment. These embryos were observed as neurodevelopment progressed from stage 12 to stage 50 (NF).
Materials and Methods:

Organism:

For this experiment, *Xenopus laevis* embryos (Xenopus Express, Brooksville Fl.) were used to study the effects of endocrine disrupting chemicals on neurodevelopment.

Animal care and maintenance:

After their arrival, *Xenopus laevis* embryos were separated from their jelly layers with Dumont No. 5 forceps (Sigma Aldrich). They were then placed in aquaria with a false bottom, in their designated EDCs. They were maintained under natural lighting, and their designated EDCs were diluted in de-chlorinated water at room temperature. Their aqua environment was changed twice a week and they were fed yeast at least two times a week once the embryos reached stage 30.

Preparation of Solutions:
Xenopus laevis embryos were incubated with diethyl phthalates (>99.5% purity), aminomethyl propanol (90% purity), and dimethicone, and bisphenol A (>99.5% purity), all obtained from Sigma Aldrich. 10µg/ml of each chemical was dissolved in 1M ethanol stock and diluted in the de-chlorinated water containing the embryos to reach a final volume of a 1:100ml solution to water ratio.

**Exposure to Endocrine Disruptors:**

For the most successful trials, Xenopus laevis embryos were divided into five (5) groups of six (6) embryos each, with one group being the control group of the experiment. They were all incubated starting at stages between 12 and 17 (taking into account all performed successful trials), the end of neurulation, and observed until approximately stage 55. The control, group 1, was only incubated in de-chlorinated water. Group 2 was incubated with diethyl phthalate, group 3 with aminomethyl propanol, group 4 with dimethicone, and group 5 with bisphenol A.

**Assays:**

The embryos were observed for differences in rate of brain development, brain size, and brain morphology based on the stages according to Nieuwkoop and Faber (NF) ([See Fig. 7], 1994). The observations under the stereoscopic microscope started at stage 13, which is characterized with a faint delimitation of the neural plate. The development and closing of the neural plate (NF stage 14 to
20) were closely observed for morphological differences in neural plate folding. The formation of the prosencephalon, mesencephalon, and rhombencephalon (NF stage 22) were compared among the groups. Additionally, the formation of the telencephalon, diencephalon, mesencephalon, metacephalon, and myelencephalon (after stage 22) were observed and compared for morphological differences. The assay also included the assessment of which endocrine disruptors were most potent during neurodevelopment. A total of five assays were performed; however only two were successful due to variable hatching success and shipping irregularities.

**Photography:**

The camera used was a Canon EOS digital camera. The photos allowed for the comparison in brain development among groups 1 to 5 of *Xenopus laevis*. They were taken twice a day for a period of two weeks. Photos were compiled from the most successful trials.

**Further observations**

Using a ruler placed on the base of the microscope attached to the Canon camera, the length of tadpoles and the width of their heads were measured starting at stages 47. The length of each tadpole was measured in centimeters from the tip of the head to the tip of the tail. The widths of the heads were also measured in centimeters from the tip of the left side to the tip of the right side.
The average length of tadpoles and width of heads was then calculated for each group.

Viability, as the days progressed, was also recorded and taken into account to determine the potency of EDCs. In addition, pigmentation differences were also observed across the groups using a stereoscopic microscope. Comparisons were also made using photos from the Canon EOS digital camera.

**Results**

**Neural plate development**

Comparing the four experimental groups incubated with diethyl phthalate, aminomethyl propanol, dimethicone, and bisphenol A, to the control group, no significant changes were observed during neural plate development. The pineal body invagination during neurulation was observed to be similar across all groups and showed normal developmental morphology. The first set of photos taken show tadpoles at different developmental stages; all of which, represent normal morphological characteristics. (see Fig. 1)
Spinal morphology

Spinal cord defects, some more severe than others, were observed in tadpoles incubated in EDCs. Straight spinal chord morphology was observed in all the tadpoles of the control groups, and this set a standard for the tadpoles incubated in EDCs. In the first successful trial, a slight bend was seen towards the distal end of the spinal cord of one of the tadpoles incubated in phthalate after stage 38. Such slight bends were also observed in three tadpoles incubated in dimethicone during another successful trial (see Fig 2.).

**Fig 2:** Slight bend at distal end of spine in tadpoles incubated in dimethicone, compared to the Control ~ St. 41
Severe spinal malformations were observed in two tadpoles, one incubated in dimethicone (see Fig 4), and the other in amino methyl propanol (see Fig 3). The curvatures became more obvious post stage 38, and their severity worsened and resulted in significant physiological defects. In the tadpole incubated in dimethicone, the curvature started proximal to the head and organs, and became more apparent near the tail after stage 43. In the tadpole incubated in amino methyl propanol, the curvature started at the distal end of the tail but became proximal to the head as it progressed in stages. These malformations caused these particular tadpoles to swim dorsally in circular patterns.

**Fig 3:** Severe curvature in tadpole incubated in amino methyl propanol
Size and Length

**Fig 4:** Severe curvature in tadpole incubated in dimethicone
In comparison to tadpoles of the control group, tadpoles incubated in EDCs were on average longer and bigger. At stage 47, the average tadpole length for the control group was 1.10cm, with a head width average of 0.30cm. The average length of tadpoles was 1.19cm in phthalate, 1.46cm in amino methyl propanol, and 1.54cm in dimethicone. The average head width of tadpoles in amino methyl propanol and dimethicone were 0.02cm larger than the average for the control (see Table 1).

**Potency of endocrine disruptors**

In the most successful trial, the viability of the control group remained 100% throughout. Of all solutions, bisphenol A was the most potent as tadpoles in that group were not viable after stage 23. Tadpoles incubated in phthalate were unresponsive in their designated solution after stage 44, thus it was second most potent. Tadpoles incubated in amino methyl propanol and dimethicone survived throughout the experimental time frame, however the survival rate in amino methyl propanol dropped to 50% by day 18, and that in dimethicone dropped to 17% by day 18. (see Fig. 5)

<table>
<thead>
<tr>
<th>Table 1: Average body and head lengths of tadpoles.</th>
</tr>
</thead>
</table>

**Fig. 6:** reduced pigmentation in tadpoles incubated in EDCs,
Additional observations

A prevailing observation consistent among all experimental groups was the reduced dorsal epithelium pigmentation of dark patches, in comparison to the control group (see Fig 6). This observation was fairly consistent among all groups; some tadpoles in fact exhibited a reddish appearance. The swimming patterns were fairly consistent among all tadpoles; except in the tadpoles incubated in amino methyl propanol and dimethicone with spinal deformities that caused them to swim in circular patterns; as they advanced in stages, their movements became highly impaired.

Discussion

Endocrine disrupting chemicals are exogenous substances with the ability to cause adverse effects to an organism and/or its progeny by altering functions of its endocrine system. Their structural similarities to hormones, such as the thyroid hormone, allow them to mimic and interfere with receptor-mediated
responses in cells. Their inability to become regulated like hormones, however, accounts for their neurotoxicity as they disturb hormonal balances in the body, including those crucial for proper neurodevelopment. These disturbances have been suggested to negatively affect sensory, motor, and cognitive functions, leading to disorders and disabilities. In this experiment, notable morphological alterations were observed as a result of *Xenopus laevis* embryos' exposure to EDCs.

Although embryonic incubations were initiated at different stages, *X. laevis* tadpoles weren't more than 3 stages apart from one another. The neural plate development was similar in all tadpoles and thus it was implied that the neurocentric canal and neural folds developed normally in all tadpoles. To infer the least, EDCs did not cause any externally obvious brain malformations during the early stages in the tadpoles incubated in EDCs, as they all maintained head morphology similar to the control group (Fig 1). Alterations, leading to the morphological abnormalities observed must have occurred at the receptor level, where genetic expression was targeted, impairing forthcoming developing characteristics such as the spinal cord. These receptor level changes could have also affected epithelial pigmentation differences observed in groups exposed to EDCs.

Spinal malformations were most prevalent in groups incubated in EDCs but the most significant were the severe curvatures observed in tadpoles incubated in amino methyl propanol and dimethicone. This curvature caused behavioral changes in the tadpole that included a circular swimming pattern and difficulty
maintaining a ventral position. These physical differences were only observed post stage 38, which is a period more associated with thyroid development than brain development. The thyroid primordium starts to develop around NF stages 33 and 34, and thyroid development continues well into stage 49 (Nieuwkoop and Faber, 1994). Since the neural plate formations were similar across all groups, it can be inferred that the neurotoxicity of dimethicone and amino methyl propanol became effective during thyroid development; not during primordial brain development. Changes must have occurred at the receptor mediated level that caused alteration in the effectiveness of thyroid hormone mediated developments.

Marsh-Armstrong et al. (2004) have actually shown that the thyroid hormone directly affects the spinal cord by regulating the generation of spinal cord cells. Interfering with the thyroid hormone function causes morphological defects in the hind limbs associated with the spinal cord. In addition, the actions of the thyroid hormone in the spinal cord are directly mediated by thyrotropin; therefore EDCs might have participated in the alteration of thyrotropin function, which further negatively affected the thyroid hormone. It can thus be inferred that the malformation seen in the tadpoles incubated with amino methyl propanol and dimethicone was due to receptor-mediated interference of the thyroid hormone, which induced a spinal malformation and impaired swimming ability. This notable evidence is suggestive of sensory and motor inhibition caused by EDCs.
As observed, tadpoles exposed to EDCs were on average larger than the control tadpoles, suggesting the disruption of growth hormones and the contribution to obesity. The literature on the effects of EDCs on GH is very scarce, and those on obesity are still under investigation. Nevertheless, GH, like the thyroid hormone, is another hormone that is regulated by the hypothalamic-pituitary axis. The ability of EDCs to effect the production of these hormones on different levels have lead to over production of the growth hormone that can account for the enlargement of tadpoles exposed to EDCs. Also, alterations of the TH hormone can be related to metabolic alterations that can affect size. Affirmatively, the thyroid areas of tadpoles exposed to EDCs were on average larger than those of the control, accounting for a bigger head width as well.

Many studies have suggested a relation between the decline of amphibians and the increase in pesticides (Hayes, 2006). Overall, exposure to EDCs presented a toxic environment to tadpoles that induced metabolic cessation and death. The toxicity of EDCs, BPA and phathlates especially, can probably induce a shock that stops all metabolic activities. Looking at this phenomenon from the hypothalamic pituitary axis, the disruption of the thyroid hormone has a significant impact on metabolism.

Nevertheless, some indications of EDC’s role in gene expression alterations were seen with the spinal malformation and dorsal epithelium dissimilarities. Future experiments are definitely anticipated in order to make better observations and monitor for consistency in the above-cited results.

Bibliography


Appendix

Figure 7: Selected Developmental Stages of *Xenopus laevis* (Niewkoop and Faber, 1994)

<table>
<thead>
<tr>
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Stage 19, dorsal view
20 hr 45 min pf @ 23°C
Stage 19, anterior view
20 hr 45 min pf @ 23°C
Stage 20, dorsal view
21 hr 45 min pf @ 23°C
Stage 20, anterior view
21 hr 45 min pf @ 23°C
Stage 21, dorsal view
22 hr 30 min pf @ 23°C
Stage 21, anterior view
22 hr 30 min pf @ 23°C
Stage 22, dorsal view
24 hr pf @ 23°C
Stage 22, lateral view
24 hr pf @ 23°C
Stage 23, dorsal view
1 day, 45 minutes pf @ 23°C
Stage 23, lateral view
1 day, 45 minutes pf @ 23°C
Stage 24, dorsal view
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Stage 24, lateral view
1 day, 2 hr 15 min pf @ 23°C
Stage 25, dorsal view
1 day, 3 hr 30 min pf @ 23°C
Stage 25, lateral view
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**Figure 8:** (NF stage ~ 32)-Tadpole incubated in amino methyl propanol with spinal curvature and enlarged thyroid area
Figure 9: (NF stage ~ 32) – Tadpole incubated in dimethicone with spinal curvature
Figure 10: Day 3 – Control tadpole vs. tadpole incubated in Bisphenol A