# THE EFFECTS OF WARFARIN AND ALPHA ENDOSULFAN ON THE THYROID HORMONE RESPONSE ELEMENT HALF SITE DR4

An Undergraduate Research Scholars Thesis

by

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# ABSTRACT

The Effects Of Warfarin And Alpha Endosulfan On The Thyroid Hormone Response Element Half Site DR4. (May 2015)

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During development, cues from the environment play an important role in directing the processes of differentiation. One really important molecule in this regard is thyroid hormone. Thyroid hormone is transported across the placental membranes and pushed into fetal circulation where it helps direct the development of the fetal brain and many other aspects of the fetal growth. Importantly, many chemicals in the environment have the capacity to alter normal thyroid hormone levels and disrupt the crucial processes regulating homeostasis. This project seeks to identify the role of two environmental toxins: warfarin, a blood thinner, and alpha endosulfan, a pesticide, to determine whether they are capable of altering neural differentiation by impairing thyroid hormone signaling. This project will investigate a thyroid hormone regulated reporter gene under the regulation of thyroid hormone response element site DR4. The goal of this research is to determine whether the addition of an environmental toxin will affect the regulation of gene expression.

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# NOMENCLATURE

DMSO ..... Dimethyl sulfoxide

DR4 ..... Direct Repeat 4

ER8 ..... Everted Repeat 8

T3 ..... Triiodothyronine

T4 ..... Thyroxine

TR ..... Thyroid hormone receptor

TRα1 ..... Thyroid hormone receptor alpha 1

TRE ..... Thyroid response element

TSH ..... Thyroid stimulating hormone

# CHAPTER I

# INTRODUCTION

## Environmental Toxins

Everyday, people are exposed to thousands of chemicals, good and bad. This can be in the form of air pollution, food, water, drugs, or exposures at work. Furthermore, if a woman is pregnant, these chemicals can be transferred to the developing fetus in utero. Many of these environmental toxins can have an effect on the body ranging from growth and development to the endocrine system. In this study, the toxins of interest are warfarin, a blood thinner, and alpha endosulfan, an insecticide.

In 1962, Rachel Carson published the book, *Silent Spring*; this book addresses environmental toxins with the present and future consequences in mind (Carson, 1962). Carson emphasizes the effects of environmental toxins on human health including the carcinogenic and mutagenic properties of pesticides (Thomson, 2013). Following Carson's mindset regarding environment and human health awareness, this research seeks to find how environmental toxins affect thyroid response sites as keys to development and everyday processes.

### *Warfarin*

Warfarin is a medically prescribed drug to help prevent blood clots and clots associated with a heart-valve replacement in addition to decreasing the chance of subsequent heart attacks. This is done by preventing vitamin K-dependent clotting factors (II, VII, IX, and X) and anticoagulant proteins C,S, and Z (Freedman, 1992). Warfarin has been shown to have an interaction with

thyroid hormone as those who experience hypothyroidism (characterized by low levels of circulating thyroid hormone) are less responsive to warfarin treatment (Stephens et al., 1989), and those who experience hyperthyroidism (characterized by high levels circulating thyroid hormone) will have an increased response to warfarin (Chute et al., 1997). Case studies involving warfarin include samples of people over 75 years of age, especially those with arrhythmia such as atrial fibrillation (Mant et al., 2007).

Furthermore, warfarin is classified as in Pregnancy Category X (FDA, 2011), meaning that it should absolutely be avoided during pregnancy. It is known that there can be congenital malformations of the fetus and an increased rate of spontaneous abortion and fetal mortality. Warfarin is able to cross the maternal-fetal blood barrier through the placenta and, if affected during the first and most susceptible trimester, can cause fetal warfarin syndrome or warfarin embryopathy. This is characterized by nasal hypoplasia, growth retardation, and central nervous system abnormalities (Hou, 2004).

*Alpha Endosulfan*

Alpha endosulfan is an organochlorine insecticide used on teas, grains, tobacco, and cottons (EPA). Although alpha endosulfan is banned in many other countries, it is still used in the United States in minimal quantities, as it is currently being phased out by 2016 due to toxicity (EPA, 2010). Alpha endosulfan is in the Toxicity Class I and is known to effect the central nervous system, as well as liver, kidney, and linked to reproductive problems in both male and females.

The EPA allows no more than 0.1 to 2.0 ppm in food other than dry tea, which is a maximum of 24 ppm (EPA). The oral LD50 for rats ranges from 18 to 160 mg/kg, but a dose as low as 2.5 mg/kg/day can exhibit teratogenic effects in rats (EXTOXNET, 1996). Alpha endosulfan is not soluble in water; however the half life is 35 days in soil. There is a higher concentration of alpha endosulfan in areas of agriculture ranging from 18 to 82 $pg/m^3$, but food is the main source of alpha endofulan exposure (Registry, 2013b). People can be exposed to alpha endosulfan by eating foods processed with the insecticide or tobacco products treated with alpha endosulfan (Mergel, 2011) ranging from .011 to .037 ppm.

If there is excessive alpha endosulfan exposure during pregnancy, development of the fetus can be altered. Effects could include the child being on the autism spectrum, effects on thyroid function, and neural tube defects (Registry, 2013a). Endosulfan exposure has been shown to directly increase T3 levels and slightly increase T4 (Freire et al., 2012).

**Thyroid Hormone**

The thyroid hormone system is a key player during pregnancy allowing for differentiation during fetal development as well as a role in metabolism. Pregnancy is a finely tuned process, with regulation at every step. There are check points, but if something slips by, then development may not occur correctly. Chemicals, including environmental toxins able to pass through the fetal barrier, can alter this finely tuned process. Thyroid response elements play a key role in development during pregnancy. Thyroid hormone, however, is also very susceptible to change and alterations. The goal of this research is to see what kind of changes happen to thyroid response elements and how that change can affect what happens during pregnancy.

During pregnancy, congenital hypothyroidism can cause deficiencies in brain development and thus a decrease in the child's IQ once the child is born (Zoeller, 2003). During development, the fetal concentration of thyroxin (T4), free T4 (FT4), and thyroid stimulating hormone (TSH) are increasing in concentration while brain development is occurring (Escobar et al., 2004). In the developing fetal nervous system, as neurogenesis is occurring, thyroid hormone levels rise accordingly to development.

Thyroid hormone also has a role with gene regulation. Previous research has shown, that T3 has an inverse relationship with sox2 gene expression through the thyroid hormone interacting with the receptors: as the TRα1 expression increases, the sox2 expression decreases (López-Juárez et al., 2012). The sox2 gene is responsible for fetal neural stem cell differentiation; however, the relationship with the thyroid hormone suggests that thyroid hormone plays a role in differentiation. This project analyzed the thyroid hormone response element (TRE) binding sites for the thyroid hormone; other receptors for thyroid hormone include DR4 and ER8.

**Mechanism**

The construct used in this experiment utilized a direct (DR4) thyroid response element. The goal of this research is to determine if the addition of the chemicals, such as warfarin and alpha endosulfan, will affect gene transcription by acting as thyroid receptor antagonists. Understanding the molecular mechanism in thyroid hormone signaling might aide in the efforts to eliminate environmental toxins from impairing thyroid signaling during development.

# CHAPTER II

# MATERIALS AND METHODS

## Cell Culture

Human Embryonic Kidney (HEK) 293 cells were obtained from ATCC (CRL-1573). The cells were grown in a humidified environment with 5% $CO_2$. Cells were grown in DMEM (11965-092) purchased from Invitrogen. These HEK 293 cells were used for all experiments.

### *Transfection*

The HEK 293 cells were transfected with either GFP, DR4, renilla constructs or TRα1, DR4, renilla constructs. The GFP plasmid has a GFP receptor downstream the promotor to report gene transcription, and is used as a control to verify transfection. The DR4 construct was developed by D Muller (D Muller et al, unpublished) and has a luciferase receptor downstream to report gene transcription. The renilla plasmid was obtained from Clontech (PRL Control) and has a renilla coding sequence downstream to report gene transcription. The TRα1 construct was developed by D Muller (D Muller et al, unpublished) and has a luciferase receptor downstream to report gene transcription.

Using Lipofectamine 2000 (11668-019) purchased from Invitrogen, transfection was completed using manufacturer's protocol.

**Treatments**

Cells treated according to Table 1 for a total of 4 trials and treated according to Table 2 for a total of 8 trials.

Table 1.

500nM Treatment of Warfarin

| | | | |
|---|---|---|---|
| GFP | GFP | | |
| DMSO 3.14µM | DMSO 3.14µM | T3 150nM | Warfarin 500nM + T3 150nM |

Legend:

| |
|---|
| GFP, DR4, Renilla |
| TRα1, DR4, Renilla |

Table 2.

1000nM Treatment of Warfarin and 1000nM Treatment of Endosulfan

| | | | |
|---|---|---|---|
| GFP | GFP | Warfarin 1000nM | Warfarin 1000nM |
| Untreated | Untreated | Warfarin 1000nM + T3 150nM | Warfarin 1000nM + T3 150nM |
| DMSO 3.14µM | DMSO 3.14µM | Endosulfan 1000nM | Endosulfan 1000nM |
| T3 150nM | T3 150nM | Endosulfan 1000nM + T3 150nM | Endosulfan 1000nM + T3 150nM |

Legend:

| |
|---|
| GFP, DR4, Renilla |
| TRα1, DR4, Renilla |

Cells were treated in duplicates. Wells were either transfected with the GFP, DR4, renilla constructs or the TRα1, DR4, renilla constructs according to the legend. The DMSO (472301-100) was obtained from Sigma and reflects the maximum possible exposure of DMSO from diluting warfarin and alpha endosulfan. The concentration of DMSO for this pair was 3.14 µM. T3 (709719-1) was obtained from Sigma and was treated in the biologically relevant concentration of 150nM. Warfarin was obtained from the EPA. Warfarin treatments were at 500nM and 1000nM. At 3.2µM to 16µM ranges of warfarin treatments of osteoblasts, warfarin has shown a biochemical effect on expression of vitamin K dependent proteins (Barone et al., 1994). Alpha endosulfan was obtained from EPA. Cells were treated with 1000nM alpha endosulfan. The $LC_{50}$ has been shown to be between 11.2µM and 48µM (Chan et al., 2006). The cells were then grown over two days and were then analyzed.

**Analysis**

GFP was observed with standard microscopy. Cell death assays were performed on the wells. Using trypan blue to stain the dead cells, the wells were observed using standard microscopy.

*Luciferase and Renilla Assay*

Using Stop and Glo obtained from Promega (E1960), a dual luciferase reporter assay was performed according to manufacturer's protocol. The plate was analyzed using a luminometer.

# CHAPTER III

# RESULTS

The luciferase and renilla readings were quantified and ratios of luciferase output to renilla output calculated. These numbers were utilized in a Wilcoxin multiple comparisons test.

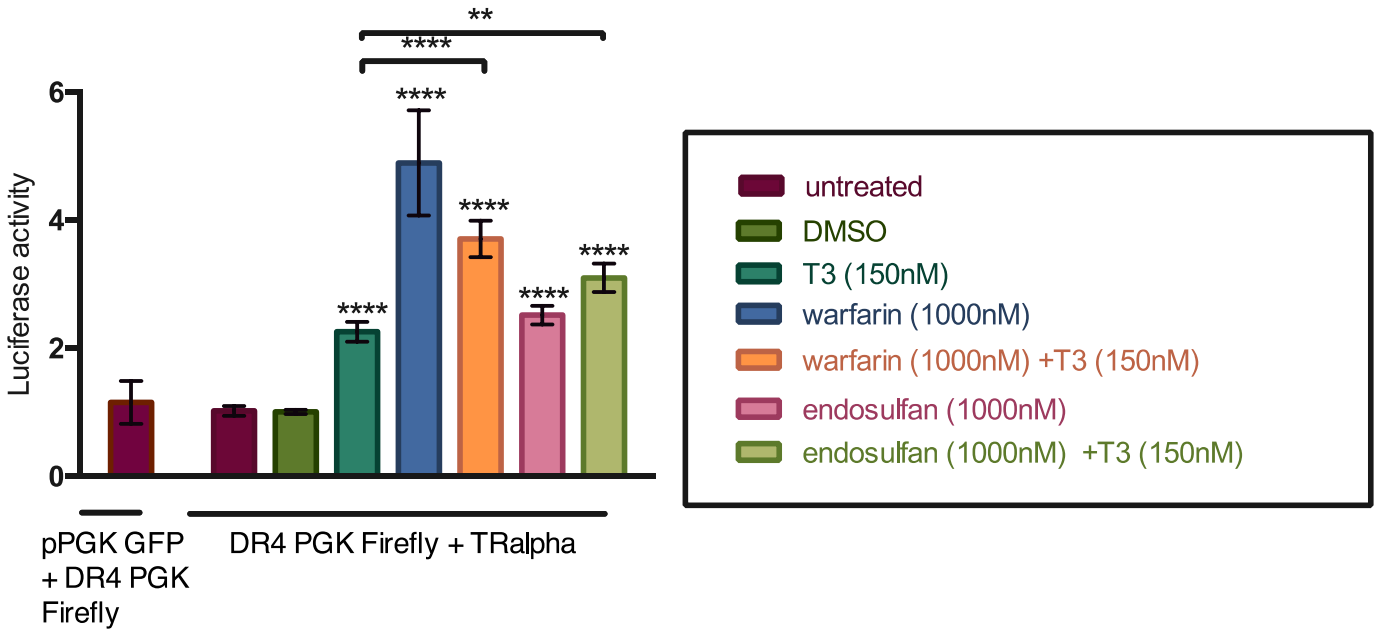


Figure 1. Results of the luciferase activity for the different treatment groups. Graph produced by Prism.

As can be seen in figure 1, there was not a statistically significant change between untreated cell and cells treated with DMSO. There was, however, a statistically significant change between untreated or DMSO cells with cells treated with T3 ($p < .0001$). This increase in expression with the addition of thyroid hormone has been seen before in research (Zoeller, 2003).

## Warfarin

Cells were first treated with 1000nM of Warfarin. There was a treatment group with just warfarin and a treatment group with warfarin and T3 in order to give the cells typical biological

developing conditions. Statistically significant differences in luciferase activity were observed between untreated cells and cells treated with 1000nM warfarin ($p < .0001$); between cells treated with T3 and cells treated with 1000nM warfarin ($p < .01$); between untreated cells and T3/1000nM warfarin treated cells ($p < .0001$); between T3 treated cells and T3/1000nM warfarin treated cells ($p < .0001$).

**Alpha Endosulfan**

Following similar procedures to the warfarin treatments, cells were first treated with 1000nM of Endosulfan. There was a treatment group with just endosulfan and then a treatment group with endosulfan and T3 in order to test the potential biological impact of this chemical. Luciferase expression was quantified in the same manner as the warfarin treatments and shown in figure 1. Statistically significant differences in expression were observed between untreated cells and cells treated with 1000nM endosulfan ($p < .0001$); between untreated cells and T3/1000nM endosulfan treated cells ($p < .0001$); between T3 treated cells and T3/1000nM endosulfan treated cells ($p < .001$); there were no statistically significant differences in expression between cells treated with T3 and cells treated with 1000nM endosulfan.

# CHAPTER IV

# CONCLUSION

## Discussion

During fetal development, stem cells need to differentiate to develop. Regulation of this differentiation is done by many molecules in fetal circulation, including thyroid hormone. Thyroid hormone is a key player in neural development and does this by binding its receptor and interacting at defined genomic binding sites such as the DR4 or ER8 elements. However, thyroid hormone is not the only molecule that can interact with at these receptors. Environmental toxins are able to pass into fetal circulation and can affect transcription. These experiements examined the interaction of warfarin and endosulfan at the thyroid hormone receptor and DR4 site to view the effects on transcription.

After analyzing the results of luciferase expression from the different treatments, several key trends were noted. First, cells treated with T3, demonstrated an increase in expression, as was expected. Interestingly T3 and warfarin were able to activate transcription to increased levels. The same trend was observed between  T3 and endosulfan treated cells.

These findings convey that exposure to warfarin or endosulfan will increase thyroid regulated expression. While the mechanism of how these environmental toxins affect expression is yet to be determined, this experiment holds promise for further studying with environmental toxins and fetal development.

Future research needs to be conducted examining a range of different concentrations of treatment groups to determine if different concentrations can effect expression at different levels. Furthermore, research can be conducted to see if the results on the DR4 site are similar to the ER8 site found in other thyroid regulated genes. Additionally, this preliminary research is limited in the fact that the experiments were done using HEK293 cells. Treatment of other cell lines or other methods of testing gene expression, in addition to just the luciferase output could give important information for this field.

## Conclusion

Environmental toxins are everywhere and can effect development as well as normal lifestyles. Being able to understand how certain toxins interact with our genes is important for pregnant women with developing fetuses and serves to help formulate regulatory control of their use. Although warfarin is supposed to be absolutely avoided during pregnancy, understanding the potential effects is still important. Additionally, endosulfan is a pesticide still used in the United States and people are exposed to this chemical; thus understanding the effects is important. Both warfarin and endosulfan have shown an effect on expression, but more research needs to be done to understand this essential topic even more.

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