Transgenerational Effects of Exposure to Bisphenol-A on Histone Acetylation of C57BL/6 Mice

By

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Allegheny College

Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the college community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. 1st Reader

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Dr. 2nd Reader

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Abstract[1]

Endocrine disruptors are an increasing problem because humans are exposed to them on a daily basis. Bisphenol-A and diethylstilbestrol are both estrogenic compounds that can disrupt the function of the endocrine system within humans. This can lead to reproductive and even behavioral problems. Endocrine disruptors can have a transgenerational effect where offspring show signs of exposure without actually being exposed to the chemical itself. Epigenetic changes may be related to transgenerational effects. Specifically, epigenetic alterations such as DNA methylation or histone acetylation can modulate transcription without changing the DNA sequence itself. Alterations of DNA methylation and histone acetylation may also cause some cancers. To investigate possible transgenerational effects, pregnant female mice were exposed to BPA and two generations of progeny were reared in absence of further exposure. Mice were prenatally exposed to BPA, DES, or EtOH (10 µg/ml) starting one week before pregnancy through weaning. Mice grew to sexual maturity and two female mice were selected from each trial and breed a 3rd generation. The remaining mice will be euthanized and tested for histone acetylation. The data did not support the hypothesis that BPA and DES would increase histone acetylation. There were no differences to overall histone acetylation between the three treatments. Over the three generations, there was only a significant increase in histone acetylation between the F1 and F2 mice for the EtOH and BPA treatment.

Introduction

Endocrine disruptors are xenobiotic chemicals that are able to disrupt normal hormone function when in contact with humans or other animals. These chemicals disrupt homeostasis in the endocrine system by manipulating the production, release, metabolism, and elimination of our natural hormones. Endocrine disruptors can even mimic natural hormones (Casals-Casas and Desvergne 2011). Some of the effects of endocrine disruptors on animals are reproductive irregularities, immune system problems, cancer, and behavioral problems (Krimsky 2000). Humans are mainly exposed to endocrine disruptors through drinking water or food supply (Tang-Péronard 2011). Two of the known endocrine disruptors are bisphenol A and diethylstilbestrol.

Diethylstilbestrol

Diethylstilbestrol (DES) is a synthetic estrogen that was prescribed to pregnant women between 1940s and 1970s to prevent miscarriages (Newbold 2005). DES was one of the first chemicals to be labeled as an endocrine disruptor. Farmers gave DES to cows in order to cause a rapid weight gain (Langston 2010). In 1971, researchers found rare vaginal cancer in many young females and DES was later found to be the cause. The mothers of the cancer patients were given DES during pregnancy. By 2002, DES was known as a carcinogen and a developmental toxicant (Langston 2010). DES exposure *in utero* disrupts differentiation in the reproductive tract which can disrupt structure or function. This can lead to tumors in hormone-bound organs (Cheng and Tang 2012).

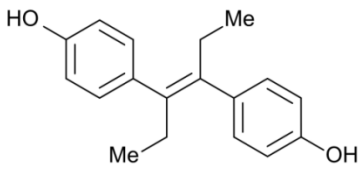


Figure 1: Chemical structure of Diethylstilbestrol.

Since DES has shown to cause rare cancer in female offspring (Langston 2010), another study looked at the grandchildren of the DES exposed women. In the 3rd generation, increased levels of cardiac problems in female offspring were reported. No such problems were found in males (Titus-Ernstoff et al. 2010). Jannerich et al. 1997 saw a two fold increase of congenital heart disease when human were prenatally exposed to an estrogenic compound. Mice were exposed prenatally to DES (2.5, 5, or 10 µg/kg per day) on days 9-16 of gestation. F1 mice were allowed to grow to sexual maturity and then were bred. The F2 mice were aged to 17-24 weeks and then examined. F1 or F2 mice were not exposed directly to DES. Both F2 males and females showed an increased number of reproductive tract tumors. The F1 lineage showed a higher overall rate (31%) of cancer than their F2 descendents (11%), (Newbold et al. 2006). These studies show the possible transgenerational effects of exposure to estrogenic compounds.

Bisphenol-A

Bisphenol-A (BPA) is an estrogenic compound that mimics estrogen and is found in polycarbonate plastics. Humans are greatly exposed to BPA because of its high production volume and wide range of uses. BPA can be found in human urine, breast milk, amniotic fluid, and placental tissues (Newbold 2010). However, BPA is shown to be in higher concentration in placental tissue and amniotic fluid rather than maternal fluids (Cheng and Tang 2012). A study conducted by the Center for Disease Control measured levels of BPA in human urine. Of the 394 adults that participated, 95% had measurable levels of BPA with an average urine concentration of 1.33 µg/L. The highest concentration found was 5.18 µg/L. Thus exposure of humans to BPA is widespread and raises concerns of the potential effects of BPA on development of humans (Calafat et al. 2005).

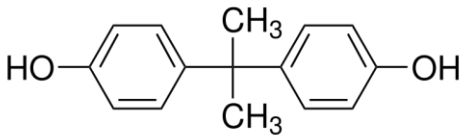


Figure 2: Chemical structure of Bisphenol-A.

Endocrine disruptors can affect offspring that are not directly exposed to the xenobiotic chemicals. How this happens is uncertain, but one of the emerging possibilities is through epigenetic changes to the genome. An epigenetic change is a change to how genes are expressed without changing the DNA sequence. Therefore, an epigenetic inheritance is the transmission of information through cell division that is not contained within the DNA sequence itself (Cooper and Hausman 2009). To change what genes are expressed without changing DNA, the mechanism that expresses them is altered.

Epigenetic Changes

DNA is wound around histone proteins to compact the DNA by about six fold which means that humans cells can store more DNA (Cooper and Hausman 2009). A normal nucleosome has five types of core histones (Bhasin et al. 2006) while histone 3 and histone 4 are the most transcriptionally active. However, compacting DNA causes one problem: the DNA needs to be unwound to be transcribed. This is done by several mechanisms including histone acetylation and DNA methylation (Cooper and Hausman 2009). Changing one of these mechanisms would lead to different genes being turned on or turned off.

Histone acetylation turns on transcription while DNA methylation turns transcription off (Cooper and Hausman 2009). This experiment will focus on histone acetylation since it activates transcription. For transcription to be activated, an enzyme, known as a HAT (histone acetyltransferase) places an acetyl group on lysine residues located on the histone proteins that are wound with DNA. This process is reversed by the enzyme HDAC (histone deacetylase), (Quenet et al. 2012). This neutralizes the positive charge in the histone proteins which relaxes the chromatin structure (Cooper and Hausman 2009). This frees the DNA from the histone proteins allowing transcription factors to bind to activate transcription.

Looking at these processes is important because environmental stimuli help shape evolution and the development of biological systems. Environmental impacts help to develop functions and biological processes within an organism. Environmental factors being able to manipulate genome activity is a

critical component of biology and evolution. In this case, estrogenic chemicals (DES or BPA) can regulate genome activity without altering existing DNA. Most environmental factors cannot manipulate DNA because the genome is very stable and is resistant to change (Skinner 2011).

Since the environmental factors can regulate genome activity, how can an epigenetic change from a toxicant pass transgenerationally? There are two types of epigenetic change; context dependent (somatic) and germ line dependent (Crews and Gore 2012). Somatic dependent is an extrinsic change and therefore needs to be present at every generation to maintain the transgenerational phenotype. A germ line change permanently alters the epigenome of the germ cell and passes onto the next generation even if the environmental factor is not present (Skinner et al. 2011).

Genomic Imprinting

Histone acetylation and DNA methylation play an important role in epigenetics because they both have a direct effect on offspring. Alterations can be made to these mechanisms in germ line cells or a modified histone can directly be transferred from parental cells to progeny cells during DNA replication. Alterations to germ line cells are most sensitive during gonadal sex determination. Primordial germ cells divide into gonadal cells such as sperm cells. Primordial cells are erased of their DNA methylation. For epigenetic changes to pass through germ line cells, they have to escape this process. Once sex determination has started, the cells develop into female or male cells and are re-methylated by genomic imprinting. This imprints epigenetic patterns to express certain genes based on sex of the germ cell. During this process, the cells can be manipulated by environmental factors (Skinner at al 2010). Genomic imprinting is more commonly regulated with DNA methylation (Cheng and Tang 2012).

Transgenerational Changes

Epigenetic marks can be changed during mitosis (germ line cells) and alter functions later within somatic cells. This means that it is possible for the epigenome to be altered by external factors during development and persist throughout life (Cheng and Tang 2012). Changes to somatic cells will not pass

to the next generation, so germ line is a critical component of transgenerational changes. During development, the genome is most susceptible to an epigenetic change (Skinner et al. 2011). During pre-natal development, when there is little hormone production, an endocrine disruptor can have a biological significance (Crews and Gore 2012). If BPA is present during this phase, modifications could be made to the histones because this is where the germ cells are most sensitive to environmental change.

Modifications to DNA or histones can be directly transferred to progeny cells. During creation of sperm, histone proteins are replaced with protamines. This alters chromatin structure. Some histones remain in the sperm cells, but DNA methylation would be the primary method of epigenetic transmission through the male germ line (Skinner et al. 2010). However, histone modifications and DNA methylation can lead to the formation of heterochromatin. These are compact chromatin structures that can remain stable through development. They will be replicated in cell division, so offspring can contain histones that have been modified in parental cells (MacDonald 2011).

Once the modified histones are in the progeny cells, they can direct modifications of newly synthesized histone proteins. Therefore, modifications to histones in the parent will directly affect histones in the offspring. This is the same case for DNA methylation; DNA methylation is stably maintained through DNA replication. A gene that has been suppressed in a parental cell remains methylated and suppressed in both progeny cells (Cooper and Hausman 2009). The mechanism of how histone marks or chromatin remodeling persist through DNA replication is currently unknown (Cheng and Tang 2012).

Post-translational modifications (PTMs) regulate chromatin structure which determines what DNA is available for gene expression. The number of post-translational modifications is less for histone 4 (H4) than histone 3 (H3) in mammals, but humans and mice have similar H3 histone modification profiles. For example, mammals have almost identical modification for the ninth lysine on H3 (Garcia et al. 2006). This is why this study will focus on H3 modifications over H4 modifications.

In human breast cancer cells, elevated levels of H3 acetylation were found (Gong et al. 2012). In mouse Leydig cells, a decreased level of acetylation was found in DES (5×10^{-8} – 5 μM) exposed cells compared to control cells. Significantly less acetylation was found in the P450scc promoter region which starts the conversion of cholesterol to steroid hormones (Warita et al. 2010). It seems that the balance between levels of acetylation and de-acetylation is more important. When one of these mechanisms is tampered with, problems can occur including cancer.

A study measured DNA methylation when A^{vy} mice were exposed to 50 mg/kg BPA through pregnancy. The offspring exposed to BPA had a change in coat color to yellow and showed a decrease in DNA methylation. Levels of methylation were similar to the brain, liver, and kidney tissue since the epigenetic effect of BPA is believed to have occurred during germ layer differentiation in the embryonic stem cells (Bernal and Jirtle 2010).

A neonatal exposure of BPA (10 μg/kg body weight) to rats showed change to gene expression in the prostate. In samples from over 30 mice, prostate tissue was screened for DNA methylation and showed to have consistent changes to an important phosphodiesterase PDE4D4. This protein is involved in controlling levels of cAMP in the prostate. Increased DNA methylation showed increased expression of this enzyme. This change is a permanent change to the genome and could even cause cancer in adult rats (Prins et al. 2008).

Pregnant mice were exposed to BPA at a low concentration (1 μg/ml) and at a high concentration (10 μg/ml). On average, the female offspring in the low dose treatment increased body weight by 13% and the high dose treatment increased body weight by 11% compared to the control group. For females, percent of adipose tissue weight to body weight was significantly higher in the LD group, but not in HD group. In males, the body weight increased by 22% in the low dose group and 59% in the high dose group. Percent of adipose tissue weight to body weight was significantly higher in the LD group, but not in HD group (Miyawaki et al. 2007).

Parental female mice were exposed to BPA through food. The offspring were exposed to BPA prenatally throughout gestation. Offspring in the first generation showed less social interaction than the control mice that were fed normal mice chow. However, in generations 2-4, the BPA treated mice had more social interaction than the control mice. The later generations were not exposed to BPA, just the first generation mother. Brain tissue was checked for estrogen receptors and the level of two hormones vasopressin and oxytocin. All BPA exposed mice had decreased levels of these hormones except only the reduction of vasopressin remained in the 4^{th} generation while oxytocin was only reduced in males in the 4^{th} generation (Wolstenholme et al. 2012).

Rats were injected with vinclozolin (100 mg/kg/day) for gestational day 8-14. Vinclozolin is a fungicide used for many crops such as grapes. Two generations of rats were breed and the 2^{nd} generations were born without direct exposure to vinclozolin. The F1 males and F2 males showed a decrease in sperm count while the F3 males did not. This study analyzed RNA strands from the male testes using a Affymetrix R230 2.0 gene chip that analyzed 30,000 gene transcripts. Compared to the controls, the F1 exposed rats had 2071 altered genes while the F2 rats had 1375 altered and the F3 had 566 altered. Most of these alterations were decreased expression of the gene while only about 10% of the changes were increased expression of the gene. The F1 and F2 mice were directly exposed to vinclozolin while the F3 rats were not. Therefore, the alterations in the F3 generation show to be a transgenerational change in expression (Anway et al. 2008).

Human breast cancer cells were treated with DES in five concentrations ranging from 5×10^{-10}M to 5×10^{-6} M and BPA in five concentrations from 2.5×10^{-8} to 2.5×10^{-4}M. Cells treated with DES and BPA showed increased levels of a protein EZH2 which regulates histone methylation. This protein is considered a gene silencer since it methylates histones which inhibits transcription. Overexpression of this protein has been seen in several human cancers including breast cancer. DES at 5×10^{-6} M showed a 4-fold increase in EZH2 expression while several concentrations of BPA saw a 2-fold increase in EZH2 expression. This suggests that BPA and DES increase levels of EZH2 by increasing translation or

decreasing protein degradation. Accordingly, increased levels of H3 methylation were found in these cancer cells (Doherty et al. 2010).

In the spring 2012 semester, I conducted an experiment in toxicology exposing African clawed frog, *Xenopus laevis*, to DES for two weeks. The frogs were injected with 3ml of DES at 5µg/ml on days 1 and 7. Frogs exposed to DES had significantly less histone acetylation than frogs exposed to mineral oil (Appendix 1). No significant difference was found between the levels of acetylation in the brain samples compared to liver samples for both treatments (Appendix 2).

Experimental Organism and Hypothesis

C57/BL-6 Jackson Lab mice were the model for this experiment since they are the inbred strain of mice used at Allegheny College. These mice are good organisms to study since they breed easily, have a long life span, and do not easily develop tumors. Based on research above, I hypothesized that both the mice exposed to DES and BPA would have an elevated level of histone acetylation. I propose that the DES mice would have a larger amount of acetylation. BPA is not as potent a chemical as DES because BPA does not have as high of a binding affinity for estrogen receptors as DES (Cheng and Tang 2012). The dosage of BPA at 10 µg/ml of water has been shown to have an endocrine disrupting effect (Miyawaki et al. 2007) which is why this dosage will be used in this experiment.

Objectives and Methods

Chemicals:

Diethylstilbestrol and Bisphenol-A were purchased from Sigma-Aldrich Chemical, St Louis MO. These chemicals were diluted to 10 µg/mL in EtOH and exposed to the mice through drinking water. Ethanol was obtained from Allegheny College and exposed to the mice as the vehicle control (1 ml/L of water). The mice were euthanized with use of CO_2 as anesthesia.

Organisms:

Six C57/BL-6 Jackson Lab mice were bred at Allegheny College. The six pregnant female mice were exposed to DES, BPA, or EtOH a week before pregnancy through weaning. After weaning, the mice were switched to untreated tap water. The treatments were:

2 Mice exposed to BPA (experimental)

2 mice exposed to DES (positive control)

2 mice exposed to EtOH (vehicle control)

A litter of 3-7 mice is expected for each pregnancy. After weaning, the mice were separated from their mothers and switched to tap water. This was approximately the 21st day of age. The mice were provided untreated Purina Rat Chow and water *ab libitum*. Their cages were cleaned and their bedding was changed 2-3 times per week. Fresh water was provided daily and the water bottles were cleaned once per week. Mice were euthanized using CO_2 after reaching sexual maturity which is approximately 5 to 8 weeks of age. Two female mice were saved from each trial to breed a third generation. Mice were not exposed to any chemicals after weaning the first generation. Brain and liver tissue was collected for examination using a histone acetylation ELISA kit. Parental mice, first and second generation mice were examined for histone acetylation. The ELISA was performed according to the manufacturer's directions. This research involves vertebrate animals and procedures were approved in accordance with guidelines and policies of the Allegheny College Animal Review Committee.

Experimental methods:

The six pregnant mice were placed into separate cages and exposed to their selected chemical through drinking water starting approximately one week before pregnancy. The mice were separated from their mothers once they were able to feed independently. Exposure of the selected chemical did not

continue after weaning. After sexual maturity, the mice were euthanized under anesthesia (CO_2). At least two female mice from each treatment were saved for breeding a third generation. The exposures were:

P (direct water exposure) → F_1 (exposed through placenta and mother's milk) → F_2 (not exposed)

Levels of histone acetylation were measured from brain and liver tissue using an ELISA kit purchased from Abnova.

Breeding Scheme:

N=28	BPA (n=12)	DES (n=5)	EtOH (n=11)
Parent	2 Female	5 Females	1 female
F1	2 females 3 males		3 females 2 males
F2	2 females 3 males		2 females 3 males

Histone Extraction:

All buffer solutions and antibodies were purchased from Abnova global histone acetylation kit. Tissue samples were weighed out and diluted with 1ml lysis buffer to 200mg of tissue. Tissue was then homogenized with 10-30 strokes and centrifuged at 3000 rpm for 5 minutes at 4^0C. Supernatant was saved and diluted with buffer at 10 µl extraction buffer to 40 mg tissue. Samples were kept on ice for 5 minutes and then centrifuged at 12000 for 5 minutes at 4^0C. Samples were then washed with TCA at 1/4the the amount of tissue (100 µl TCA for 300 µl supernatant). Samples were spun at 12000 rpm for 10 minutes and the supernatant was discarded. Samples were washed with acetone containing .1% HCL, spun at 12000 rpm for 1 min, and the supernatant was discarded. Repeat wash with just acetone. Spin at 12000 rpm for 2 minutes and remove as much supernatant as possible. Distilled water was added to the

samples at 10 µl of water to 40 mg tissue. Samples were frozen at -80^{0}C until needed for acetylation detection.

Histone H3 Acetylation Detection:

All buffer solutions and antibodies were purchased from Abnova global histone acetylation kit. Samples were removed from the freezer and diluted with a wash buffer and distilled water. 60 wells of a 96 well plate were used. Tissue was diluted with a histone buffer at 200 ng/µl. 5 µl of the sample was placed into an individual well for 56 wells. 2 positive controls used and 2 negative controls were used resulting in 60 wells used total. Wells were incubated at 37^{0}C for 90 min. 150 µl of blocking buffer was added to the wells and they were incubated at 37^{0}C for 30 min. 50 µl of diluted capture antibody was added to wells and they were incubated at room temperature on the orbital shaker. Wells were washed 3 times and 50 µl of detection antibody was added to each well. Wells were incubated at room temperature for 30 minutes and then washed 3 times. 100 µl of developing solution was added to the wells and they were incubated at room temperature for 3 minutes. 50 µl of stop solution was added and the absorbance was measured on a microplate reader at 450 nm.

Data Analysis

Differences among histone acetylation were analyzed using a one way analysis of varience (ANOVA). Graphs were produced by using a multiple comparison test (Statview 5.0, SAS Institute, North Carolina). Significant data was measured at P = 0.05 or below.

Results

Mice Breeding

When exposed to DES, the female mice failed to breed. Initially, when the two mice that were purchased from Jackson Lab failed to breed, new males were placed into the cages and DES was removed from the water. These mice still failed to breed while just on tap water. Three more inbred C57 female mice were acquired from Allegheny College for breeding. For the first two females, they were exposed to DES at half the concentration (5 µg/mL) as before. These two females still failed to breed. For the last female, food was dosed with DES at 5 µg/gram of food and the female still did not breed. At this point, offspring in the F2 generations were born for EtOH and BPA, so DES was removed from the experiment. The 5 DES females were still euthanized and tissue was extracted for histone analysis.

Histone Acetylation

For the histone acetylation, higher absorbance on the microplate reader corresponds to higher histone acetylation. Between the two litters of F1 EtOH mice, there was no difference in histone acetylation (Figure 3). For the brain tissue, the second litter showed higher acetylation, but it was not significant. Of the other trials, there was only one litter per generation, so EtOH had the only generation with two litters.

Overall, sex did not have significant differences on histone acetylation (Figure 4). For the liver tissue, the female mice showed higher acetylation in the liver tissue, but this result was not significant. There were no male mice in this result for the DES trial because only females exposed to DES were saved for histone analysis.

For overall histone acetylation (Figure 5), there were no significant differences between treatments. The DES acetylation in the liver trial showed to be higher, but it was not significant. Only females were used for the DES trials. Therefore, the DES trial had a smaller sample size (n=5) than the BPA (n=12) and the EtOH (n=11).

When measuring generation and treatment, there were no significant results except a significant difference in histone acetylation of the brain tissue between the F1 and F2 mice (Figure 6). This trend is for both the BPA and EtOH mice. Only 1 EtOH parent was used, so only the F1 and F2 mice can be compared. DES could not be compared because there were no F1 or F2 mice in the DES trial. In the liver samples, the parents appear to have higher acetylation, but it is not significant. Since only 1 EtOH female was present in the EtOH parental trial, this trial cannot be compared to the F1 or F2 trials.

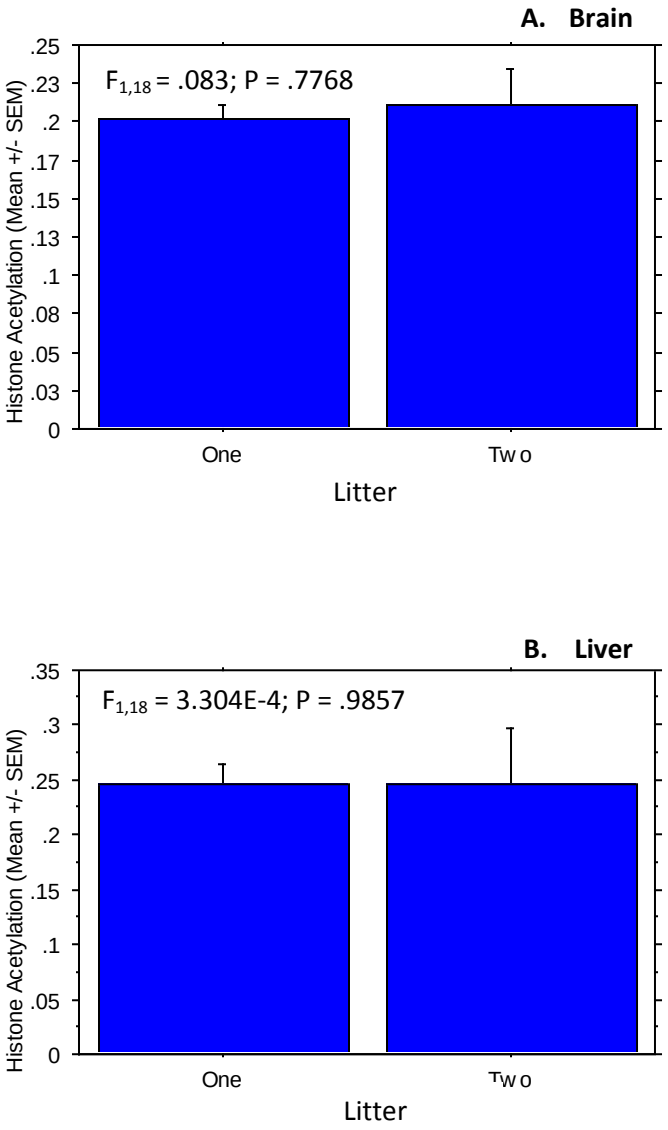


Figure 3: Absorbance at 450 nm. Mean histone acetylation (+/- SEM) of different litters (n=5) of mouse brain (A) and liver tissue (B) as measured by an ELISA. Different litters of the F1 ethanol mice are shown in this figure. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected at 5-8 weeks of age.

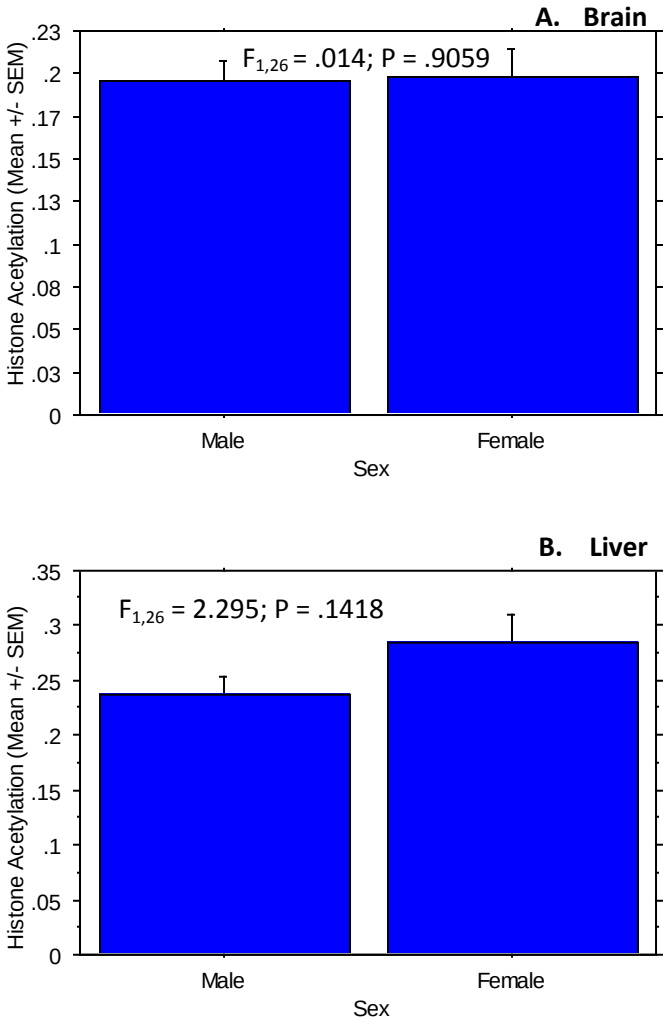


Figure 4: Absorbance at 450 nm. Mean histone acetylation (+/- SEM) of the differences between the sexes of mouse brain (A) and liver (B) tissue as measured by an ELISA. No parental males were used (n=28); only F1 and F2 males were used. All females were used including parental females. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected at 5-8 weeks of age.

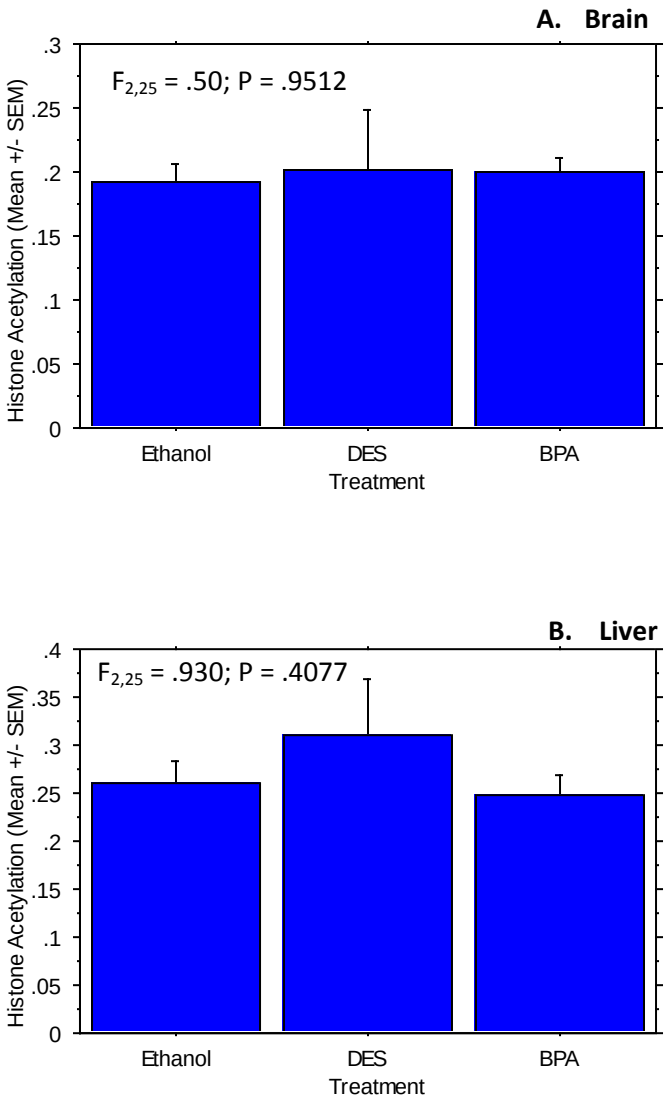


Figure 5: Absorbance at 450 nm. Mean histone acetylation (+/- SEM) of different treatment groups of mouse brain (A) or liver (B) tissue as measured by an ELISA. Overall population of mice (n=28) were used; n=11 for ethanol, n=5 for DES, and n=12 for BPA. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected 5-8 weeks of age.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Gen	1	.011	.011	9.658	.0068	9.658	.844
Treatment	1	1.300E-4	1.300E-4	.112	.7419	.112	.061
Gen * Treatment	1	4.205E-5	4.205E-5	.036	.8513	.036	.054
Residual	16	.019	.001				

Table 1: ANOVA table of the effect of treatment on generation of histone acetylation in mice brain tissue as measured by an ELISA. Only F1 and F2 data are shown since only one parental female mouse was used in the ethanol trial. No DES data is present because DES only had parental female mice. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected 5-8 weeks of age.

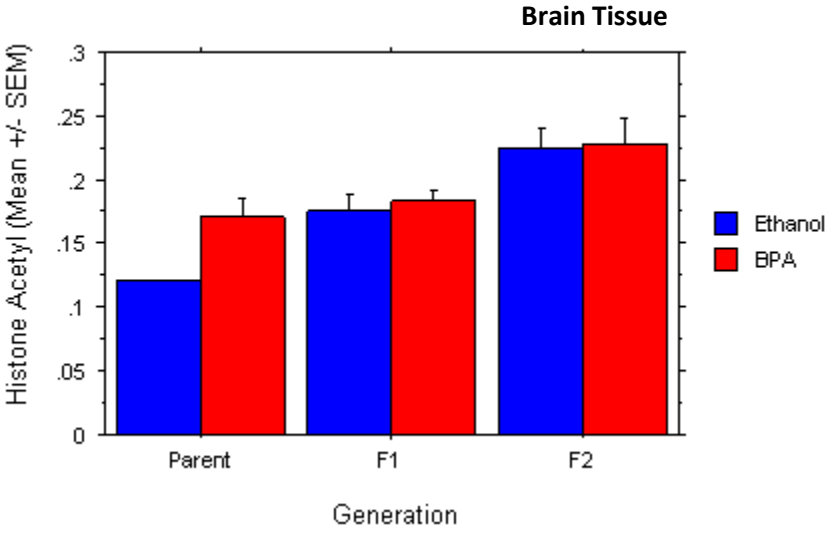


Figure 6: Mean histone acetylation (+/- SEM) of treatment on generation in mice brain tissue as measured by an ELISA. No DES data is present because DES only had parental mice. Only 1 ethanol female was used, so data were duplicated to produce an average value for parental ethanol trial. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected 5-8 weeks of age.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Gen	1	.001	.001	.181	.6762	.181	.068
Treatment	1	4.802E-4	4.802E-4	.084	.7759	.084	.058
Gen * Treatment	1	.005	.005	.849	.3704	.849	.134
Residual	16	.092	.006				

Table 2: ANOVA table of the effect of treatment on generation of histone acetylation in mice liver tissue as measured by an ELISA. Only F1 and F2 data are shown since only one parental female mouse was used in the ethanol trial. No DES data is present because DES only had parental female mice. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected 5-8 weeks of age.

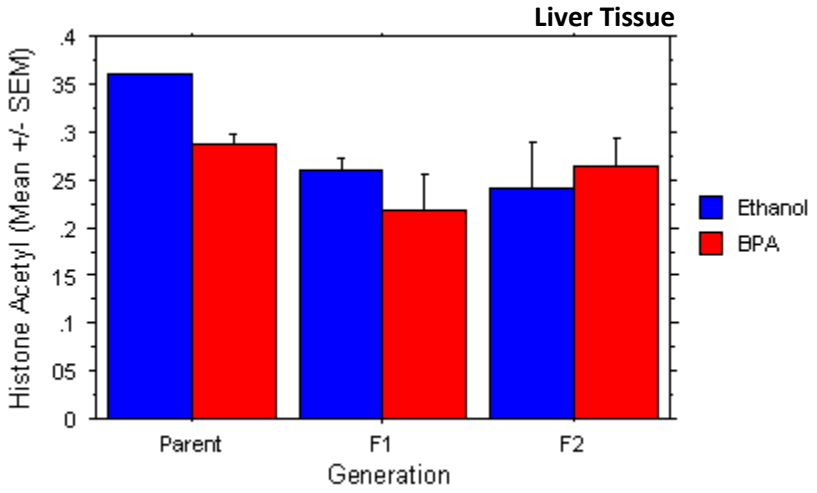


Figure 7: Mean histone acetylation of treatment on generation in mice liver tissue as measured by an ELISA. No DES data is present because DES only had parental mice. Only 1 ethanol female was used, so data were duplicated to produce an average value for parental ethanol trial. F1 Mice were treated prenatally through gestation and F2 mice were not treated. Samples were collected 5-8 weeks of age.

Discussion

The purpose of this experiment was to test if BPA has an effect on histone acetylation in mice over multiple generations. Since genome activity is regulated by environmental factors, endocrine disruptors have shown to manipulate histone acetylation in mice (Warita et al. 2010). The mechanism of how this is done is currently unknown. However, if somatic cells are exposed to environmental factors, any changes will not be passed onto the next generation. The same stressor needs to be present in the next generation for the offspring to be effected. If germ line cells are manipulated by an environmental factor, a transgenerational effect can occur where the progeny do not need to be exposed to the same stressor. A chemical that alters the germ line cells will permanently alter the genome and will pass that change onto the next generation (Skinner et al. 2011). During genomic imprinting, all cells are stripped of DNA methylation and are re-methylated during sex determination. At this time, the cells can be manipulated by environmental factors (Skinner et al. 2010). With DES as the positive control, I hypothesized that BPA and DES would increase histone acetylation in mice with DES increasing histone acetylation more than BPA. The results from this experiment did not show that BPA or DES significantly increased histone acetylation over multiple generations.

Histone Acetylation

Overall, there was no significant difference in the amount of histone acetylation in the different treatments. The liver tissue shows slight increase in acetylation, but it was not a significant result (Figure 4). Only one generation was present for the DES trial, so if the F1 and F2 generations were present, different results may have occurred.

With the ELISA kit, the manufacturer included a positive control test to make sure the kit is properly detecting histone acetylation level. In this experiment, two of the manufacturer's positive controls were placed in two separate wells and run through the acetylation detection portion of the ELISA procedure. Both of the wells with the positive control detected a proper amount of histone acetylation

according to the manufacturer's standards. Therefore, the ELISA kit was functioning properly and did not affect the results of this experiment.

In the brain tissue, over two generations, a significant difference was found in acetylation between the F1 and F2 generations (Figure 6). Since the F2 generation was not directly exposed to BPA, epigenetic changes should not be as large as direct exposure. When rats were exposed to vinclozolin and two generations were born, the first generation showed a higher percentage of tumors than the second generation. The parental line had the highest amount of affected genes, followed by the F2, and lastly the F3 strain (Anway 2008). Therefore, the mice directly exposed to BPA should have had the largest change to histone acetylation compared to control mice.

This difference in histone acetylation of the F2 generation in the brain tissue was not just the BPA trial; it also occurred in the control trial (Figure 6). The F2 generation had a significantly increased amount of histone acetylation compared to the other generations (Figure 6). Therefore, the chemical does not appear to be the cause of the increase in histone acetylation. The third generation of mice was euthanized right at sexual maturity (7 weeks of age). In the parental generation, stock mice from Jackson Lab were purchased at sexual maturity. Then they were bred and euthanized after the pups could feed on their own. During this extra time period they were not exposed to the treatment chemical. The increased age and lack of treatment during this time period could have change the acetylation patterns.

In the liver tissue, there was no significant difference between the histone acetylation of the three generations. The parents had the highest histone acetylation which decreased in the second generation. In the third generation, the histone acetylation increased in the BPA trial and slightly decreased in the control trial. This trend line was not statistically significant (Figure 7). I would propose that the parental and F1 generation to have the highest histone acetylation because they are directly exposed to the treatment chemical and the F2 generation to have decreased histone acetylation because it is not directly exposed. However, in both the brain and liver tissue, when exposed to BPA, the F2 generation increased

histone acetylation from the F1 generation, but this is not significant (Figure 6,7). BPA may increase histone acetylation over two generations, but this could be the result of euthanizing the F2 generation younger than the F1 and parental mice.

Overall, BPA with a low dose of 10µg/ml in water has shown to be an endocrine disrupting chemical in mice (Prins et al. 2008, Miyawaki et al. 2007). At low doses, BPA can be biologically active in mice and humans (Cheng and Tang 2012). In this study, BPA did not significantly alter histone acetylation levels. A larger dose may be needed to alter this mechanism because BPA is only an estrogen mimic and not a synthetic estrogen such as DES. BPA may not even alter this mechanism. There are other epigenetic processes such as DNA methylation that could be the target of BPA. DNA methylation plays a larger role in genomic imprinting which could be manipulated if BPA or DES is present during this process.

Complications

The histone extraction procedure for the liver and brain were exactly the same, but performing the procedure on the liver tissue was more consistent. When extracting the liver tissue from the mice, a 200 mg sample was easy to obtain consistently. In the brain tissue, not every extraction produced a 200 mg tissue sample. When going through the histone extraction procedure, buffers are added to the tissue according to the amount of tissue present. In the liver trials, each sample was 200 mg, so the whole procedure was consistent. In the brain trial, dilutions had to be calculated according to the amount of tissue which was not the same in every sample. This leaves more room for error in the extraction of the brain tissue. I would conclude that the liver tissue data should be more accurate because the procedure was more consistent and had less room for error.

The main limit of this experiment was the DES mothers not being able to have offspring. DES is a synthetic estrogen and would bind estrogen receptors more rapidly than BPA (Cheng and Tang 2012).

Therefore, DES is a more potent molecule than BPA. Only parental mice were examined in the DES trial. Without the F1 and F2 DES mice, data from the DES trial cannot show if there was a transgenerational effect. The acetylation in the parental mice can be compared to the other treatments, but the point of this experiment was to see if these chemicals alter histone acetylation in germ line cells and pass changes onto the next generation.

With only one euthanized mother in the EtOH trial, the parental exposure to the control cannot be compared to the other treatments. At least two mice are needed to produce an average that can be compared to other trials. Without these data, a clear trend line cannot be established from parent to F2 in the control trial. Therefore, only the F1 and F2 mice in the EtOH trial can be compared to the other treatments.

Future Experiments

Since no significant results were found with histone acetylation, I would look at other epigenetic mechanism including DNA methylation. During gonadal sex determination, primordial cells are stripped of their DNA methylation and are re-methylated according to different characteristics such as determining sex. During this process, environmental factors can alter the methylation patterns (Skinner et al. 2010). Since DES has been shown to have transgenerational effects (Langston 2010, Newbold et al. 2006), it would be interesting to look at DNA methylation as an endpoint. DNA methylation appears to play a bigger role than histone modifications in genomic imprinting (Skinner et al. 2010), which would be interesting to see if BPA could manipulate this process and pass changes to the next generation.

In this experiment, all of the mice exposed to DES failed to give birth. Another experiment would be needed to find the proper dose to allow DES exposed mice to have offspring. One option would be to wait until the mice were pregnant. Doing so would not affect the mice's ability to become pregnant while still altering germ line cells. A specific time frame within pregnancy could be targeted such as gestational days 8-14 as seen previously in (Anway et al. 2008.)

Literature Cited

Anway M and Skinner M. 2006. Epigenetic transgenerationl actions of endocrine disruptors. Endocrinology 147: S43-S49.

Bernal A and Jirtle R. 2010. Epigenomic disruption: the effects of early developmental exposures. Birth Defects Res A Clin Mol Teratol. 88: 938-944.

Bhasin M, Reinherz E, and Reche P. 2006. Recognition and classification of histones using support vector machine. Journal of Computational Biology 13; 102-122.

Calafat A, Kuklenyik Z, Reidy J, Caudill S, Ekong J, Needham L. 2005 Urinary concentrations of bisphenol a and 4-nonylphenol in a human reference population. Environmental Health Perspectives 113: 391-395.

Casals-Casas C, Desvergne B. 2011. Endocrine disruptors: from endocrine to metabolic disruption. The Annual Review on Physiology 73: 135-162.

Cheng R, Tang W. 2012. Environment, Epigenetics, and Diseases, pp 6-7, 12-13. In Sahu S (editor), *Toxicology and Epigenetics.* Wiley, NY.

Cooper G and Hausman R. 2009. The Cell: A Molecular Approach 5th Edition. ASM Press.

Crews D and Gore A. 2012. Epigenetic synthesis: a need for a new paradigm for evolution in the contaminated world. F1000 Biology Reports 4:18.

Doherty L, Bromer J, Zhou Y, Aldad T, Taylor H. 2010. In utero exposure to diethylstilbestrol (DES) or bisphenol-a (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer 3: 146-155.

Garcia B, Hake S, Diaz R, Kauer M, Morris S, Recht J, Shabanowitz J, Mishra N, Strahl B, Allis C, Hunt D. 2006. Organismal differences in post-translation modifications in histones H3 and H4. The Journal Of Biological Chemistry 282: 7641-7655.

Gong Y, Li Y, Abdolmaleky H, Li L, Zhou J. 2012. Tanshinones inhibit the growth of breast cancer cells through epigenetic modification of aurora a expression and function. PLoS ONE 7(4) : e33656. doi:10.1371/journal.pone. 0033656

Janerich D, Dugan J, Standfast S, Strite L. 1977. Congenital heart disease and prenatal exposure to exogenous sex hormones. Br Med 1: 1058-1060.

Krimsky, S. 2000. *Hormonal chaos: the scientific and social origins of the environmental endocrine hypothesis*. The John Hopkins University Press.

Langston, N. 2010. *Toxic bodies: hormone disrupters and the legacy of DES.* Yale University Press.

MacDonald W. 2012. Epigenetic mechanisms of genomic imprinting: common themes in the regulation of imprinted regions in mammals, plants and insects. Genetics Research Interational 585024.

Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H. 2007. Perinatal and postnatal exposure to bisphenol a increases apidose tissue and serum cholesterol level in mice. Journal of Atherosclerosis and Thrombosis 14: 245-252.

Newbold, R. 2010. Impact of environmental endocrine disrupting chemicals on the development of obesity. Hormones 9: 206-217.

Newbold R, Padilla-Banks E, Jefferson WN. 2006. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations.Endocrinology 147: S11-S17.

Newbold RR, Padilla-Banks E, Snyder RJ, Jefferson WN. 2005. Developmental exposure to estrogenic compounds and obesity. Birth Defects Res A Clin Mol Teratol 73: 478-480.

Prins G, Tang W, Belmonte J, Ho S. 2008. Developmental exposure to bisphenol A increases prostate cancer susceptibility in adult rats: epigenetic mode of action is implicated. Fertil Steril. 89.

Quenet D, Walkiewicz M, Dalal Y. 2012. Chromatin at the intersection of disease and therapy, pp 54. In Sahu S (editor), *Toxicology and Epigenetics.* Wiley, NY.

Skinner M, Manikam M, and Guerrero-Bosagna C. 2011. Epigenetic transgeneration action of endocrine disruptors. Reproductive Toxicology 31: 337-343.

Skinner M, Manikkam M, and Guerrero-Bosagna C. 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. Trends Endocrinol Metab. 21(4): 214–222.

Tang-Péronard J.L., Andersen H.R., Jensen T.K., Heitmann B.L. 2011. Endocrine-disrupting chemicals and obesity development in humans: a review. Obesity Reviews 12: 622-636.

Titus-Ernstoff L, Troisi R, Hatch E, Palmer J, Hyder M, Kaufman R, Noller K, Hoover R. 2010 Birth defects in the sons and daughters of women who were exposed *in utero* to diethylstilbesterol (DES). Int J Androl. 33: 337-384.

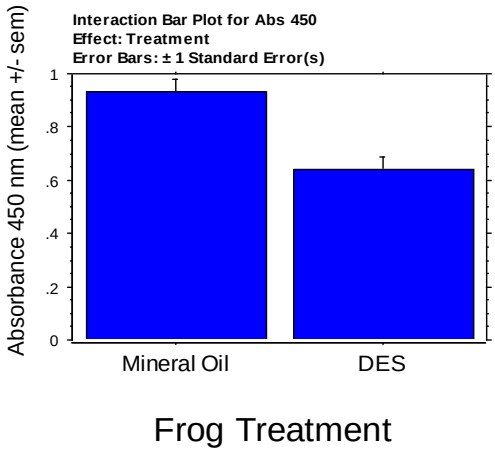
Warita K, Mitshuhashi T, Sugawara T, Tabuchi Y, Tanida T, Wang Z, Matsumoto Y, Yokoyama T, Kitagawa H, Miki T, Takeuchi Y, Hoshi N. 2010. Direct effects of diethylstilbestrol on gene expression of the cholesterol side-chain cleavage enzyme (P450scc) in testicular Leydig cells. Life Sciences 87: 281-285.

Wolstenholme J, Edwards M, Shetty S, Gatewood J, Taylor J, Rissman E, Connelly J. 2012. Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression. Endocrinology 153: 3828-3838.

Appendix 1: Overall levels of histone acetylation in DES exposed frogs compared to mineral oil exposed frogs. This is both brain and liver tissue.

ANOVA Table for Abs 450

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	.343	.343	19.066	.0006	19.066	.989
Residual	14	.252	.018				



Appendix 2: Levels of histone acetylation in frogs exposed to DES compared to frogs exposed to mineral oil. This figure examines the difference between the treatments and the tissues of the exposed frogs.

ANOVA Table for Abs 450

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	.343	.343	22.429	.0005	22.429	.995
Tissue	1	6.250E-8	6.250E-8	4.093E-6	.9984	4.093E-6	.050
Treatment * Tissue	1	.068	.068	4.469	.0561	4.469	.485
Residual	12	.183	.015				

