ANDROGEN RECEPTOR FORMATION IN PRENATALLY ENDOCRINE DISRUPTED MICE

An Undergraduate Research Scholars Thesis

by

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Approved by

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ABSTRACT

Androgen Receptor Formation in Prenatally Endocrine Disrupted Mice. (May 2014)

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Inactivity is one of the largest detriments in our society today. It has been estimated that about $434 billion are spent on inactivity related health problems in the United States per year (1). This dollar amount is only exceeded in gravity by the death toll, with more than 250,000 deaths per year directly attributing to physical activity (2). These alarming statistics have directed our lab into researching genetic factors contributing to physical activity. A current proposed mechanism contributing to physical activity suggests that androgen receptors are key mediators in activity regulation (3,4). Current literature suggests that certain chemicals, like Benzyl butyl phthalate (BBP), disrupts androgen receptor formation prenatally (5, 6) and we are hypothesizing that this androgen receptor malformation may decrease physical activity after birth. Therefore, the purpose of this study is to explore the relationship between endocrine disruptors given prenatally, androgen receptor formation, and physical activity.

CHAPTER I

INTRODUCTION

Physical activity has been a subject of interest in recent American culture. It seems everyone from Oprah Winfrey to Michelle Obama have something to say about America's need for, or lack of, physical activity. With the advent of new leisure technology and the increase in white-collar jobs the importance of physical activity has been diminished. This decline in physical activity has been matched with campaigns such as Michelle Obama's *Let's Move* campaign or the National Football League's *NFL Play 60* initiative. These campaigns have been created in order to raise awareness for the need of physical activity in Americans', especially young Americans', daily lives. While these initiatives are a great start, the need for additional measures still exists. As stated earlier, physical inactivity contributes to 250,000 deaths and about $434 billion in expenses per year (1,2). The statistics are alarming and have been some of the major reasons for research aiming to understand the factors contributing to physical activity. Physical activity is an extremely complex phenomena controlled by a variety of factors. Among these factors are genetic controls with recent research showing that physical activity has a significant genetic regulation and that predisposition to a particular activity level can be an inherited trait (2). At this time, it is still not clear which genetic/biological mechanisms are primary controllers of physical activity. It has been shown that sex hormones are a potential causative mechanism for physical activity and that supplementation of both male and female animals with testosterone while blocking estrogenic pathways, significantly increases physical activity (3,4). This work, along with other research, strongly suggests that androgen receptors – the receptors that testosterone interacts with in the body – may be mediators of physical activity.

Distressingly, it has also been discovered that common environmental chemicals such as Benzyl butyl phthalate (BBP), which is common in consumer rubber products, can act as endocrine disruptors and can disrupt the sex hormone pathways, especially in prenatal conditions (5,6). While this research has shown that these hormones have an effect on humans, the gravity of that effect has yet to be understood. Therefore, the purpose of this study was to determine if the administration of BBP to pregnant mice, will decrease androgen receptor formation in the resulting offspring.

CHAPTER II

METHODS

Sample Collection

The mice used as breeder pairs in this study were inbred C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME). Four breeder pairs of C57Bl/6J mice were purchased from Jackson Laboratories and were mated. At confirmation of pregnancy (appearance of vaginal plug), the male mice were removed from the cage. Beginning at nine days of pregnancy, the treatment pregnant mice were given interperioteneal injections of BBP (500 mg/kg) until day 16 of gestation. The control mice were injected with an equal volume of saline from days 9-16 of gestation. The resulting offspring (n=6, males=4, females=2) were weaned at 21 days of age, and housed individually with a running wheel at 4 weeks of age. The mice were sacrificed at 10 weeks of age by anesthetizing using vaporized isoflurane followed by cervical dislocation. The soleus (slow twitch skeletal muscle) was removed and flash-frozen for later analysis (7). All procedures in this project were part of a larger project approved by the Texas A&M Institutional Animal Control and Use Committee (approval 2012-0274).

Protein Extraction

To determine whether the prenatal exposure to BBP had altered androgen receptor formation, we conducted western blot analyses in the soleus tissue. The first step in the process was to extract the androgen receptor protein. To do this Dangott Lysis Buffer was made and 500 µl of buffer was then added to a chosen sample (<100 µg) in a labeled 1.7 ml tube. If sample was greater than 100 µg, 1000 µl of the buffer was added in combination with the sample. A mortar and

pestle, which had been previously placed in the freezer, was removed and a small amount of liquid nitrogen was added to the mortar. The sample was added and ground until it was a fine powder. Using an autoclaved spatula the sample was placed into the previously made 1.7 ml tube containing the buffer solution. The sample was placed on ice for 1 hour with intermittent bouts of vortexing every 15 minutes. The sample was then centrifuged for 30 minutes and 4 ºC. After centrifuging, the supernatant (protein) was removed and placed into newly labeled tubes. These tubes were placed in -20 ºC freezer for later analysis.

Protein Concentration

The concentration of androgen receptor protein – needed for loading the proper amount of sample into the western blotting - was determined using the Bradford Protein assay. Using a 96 well plate, 300 µl of coomassie brilliant blue was pipetted into (7) wells. Five additional wells were added for the five different standard Bovine Serum Albumin concentrations (2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml). A dilution series was made for each sample using *Neat, 2X, 4X, 8X,* and *16X* dilutions. Ten µl of the sample, dilutions, and standards were added to the wells containing the 300 µl of coomassie. A blue color change was observed. The Nanodrop spectrophotometer (ThermoScientific, MA) and Bradford software were used with 2 µl of each sample to determine the protein concentration.

Western Blot

Determining the actual amount of androgen receptor protein was done using a standard western blot technique. Briefly, the glass and aluminum plates (as well as the black spacers) that make

up the gel caster were placed on a clean surface. After they were spread out they were sprayed with 100% methanol and wiped dry with a lab tissue.

The black spacers were then inserted on the sides of the plates. Each gel plate pair was then inserted in the gel stand vertically. The plastic screws were then screwed in their respective holes to hold the plates in place. A gel seal was then added to the exposed corners in order to create a tight vacuum. The gel stand was then placed in the gel caster unit. Clamps were then placed on each side of the unit including the top of the gel plate and aluminum backing plate.

Next a running gel and a stacking gel were made using a pre-prepared mixture to form the agarose gel matrix. The running gel was then added between the gel surfaces using a pipette to a point approximately ¾ the height of the plate surface. In order to flatten the gel uniformly water saturated isobutanol was added to fill the remaining ¼ of the space. The gel was left to harden overnight. After it had hardened the isobutanol was poured off and the gel was washed with distilled water. The stacking gel was pipetted in between the two plates all the way to the top (remaining ¼).

After the running gel solidified and the stacking gel was poured, a 10 well comb was placed in between the plates in the stacking gel portion of the plate. After the gel had hardened the comb was removed and the wells were washed with distilled water.

Each sample was then prepared using a precise ratio of distilled water and Laemmli buffer. The sample was vortexed and incubated at 90 ºC for 12 minutes. After each sample was prepared the

gel plates were clipped to the buffer chamber. The buffer chamber was filled with “electroblot” buffer and each sample well was filled with its corresponding sample. The buffer chamber was then connected to a 25 mA current at constant voltage.

After the protein marker (blue line) reached the bottom of the gel plates the PVDF (polyvinylidene difluoride) membrane was sprayed with methanol and placed in a plastic container containing transfer buffer. Next, the gel was removed and placed in another plastic container with transfer buffer. The gel and membranes were then rocked for 20 minutes. Following this, two sponges and four pieces of filter paper were dipped in transfer buffer. The gel and the membrane were then placed very carefully in between these items with two sponges and two pieces of filter paper on each side (fig. 1). The product was then clamped and placed in the blotting chamber.

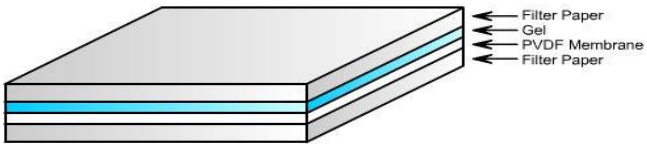

Fig 1

The chamber was then filled with transfer buffer. In order to allow a constant flow of buffer a stir bar was added. The chamber was connected to a 100 mA current at constant voltage for two and a half hours.

After the two and a half hours, the membrane was removed and placed in a plastic container containing blocking buffer. The membrane was rocked for an hour followed by three separate bouts of washing with TBS (tris-buffered saline) for five minutes.

Antibodies were applied using a dilution of 1:500 (if sample greater than 40 µg) or 1:250 (if less than 40 µg). A plastic pouch was made with the gel sheet inside. Primary anitbodies were then pipetted into the pouch. The pouch was then sealed and rocked for a few minutes and left in the 0°C refrigerator overnight.

The pouches were removed and the samples washed in TBS using the same procedure as stated previously. The secondary antibody was then prepared using a ratio of 1:2000 (if sample greater than 40 µg) or 1:1000 (if less than 40 µg). Five mL of blocking buffer was added along with the dilution of secondary antibody and pipetted into pre-made plastic pouches. These pouches were then sealed and incubated for one hour on the rocker. The samples were removed and washed with TBS using the same procedure as stated above. Using the West Pico Kit (chemiluminescence), 7 mL of liquid from both bottles were pipetted onto the membrane.

The membrane was transferred to a plastic sheet and set inside the flourchem chamber (model and manufacturer name, city). The program (which program?) was turned on and the on-screen instructions were followed with the final outputs being a captured image of the blots and a measurement of optical density of each blot.

Optical densities of all blots were compared between groups using ANOVA with treatment (BBP-prenatally or saline-prenatally). Significance was set at $p<0.05$ *a priori.*

CHAPTER III

RESULTS

Due to difficulties with the western blotting, at the time of this writing, samples from only a subset of the experimental animals had been completed (we anticipate completion of all samples by 4/30/14). In this subset, androgen receptor expression was not different between groups (Figure 2). While not in the scope of this project, our results were supported by the lack of difference in physical activity observed between the treatment groups (Figures 3 and 4). Males did not demonstrate a change in activity between treatment groups (p=0.9493; Figure 3), nor did the females (p=0.7235; Figure 4).

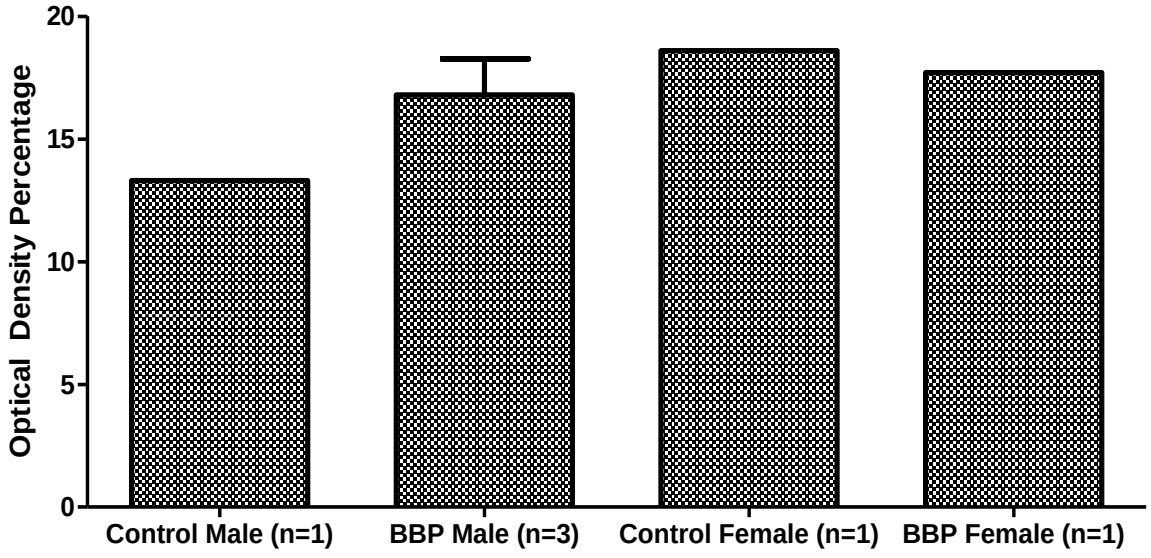

Figure 2: Band optical density comparing BBP vs control groups (and male and female within each group) These results are limited due to the small samples sizes completed at the time of this document completion.

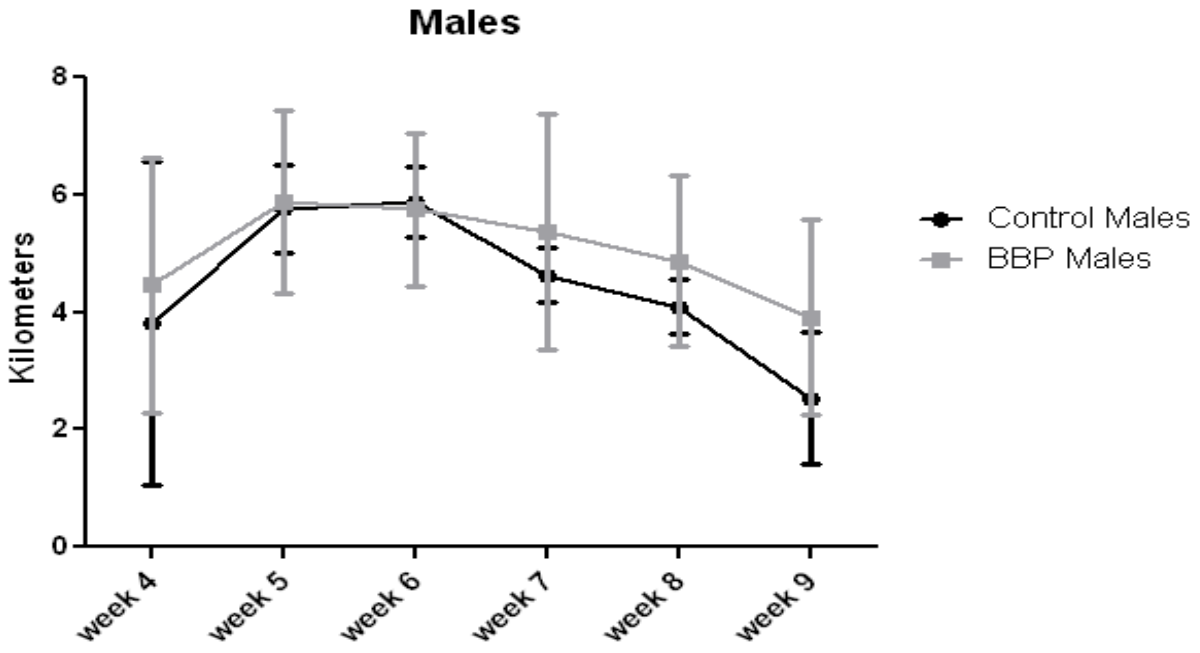

Figure 3: Activity data in males indicating no difference in running wheel distance throughout the study (p=0.9394).

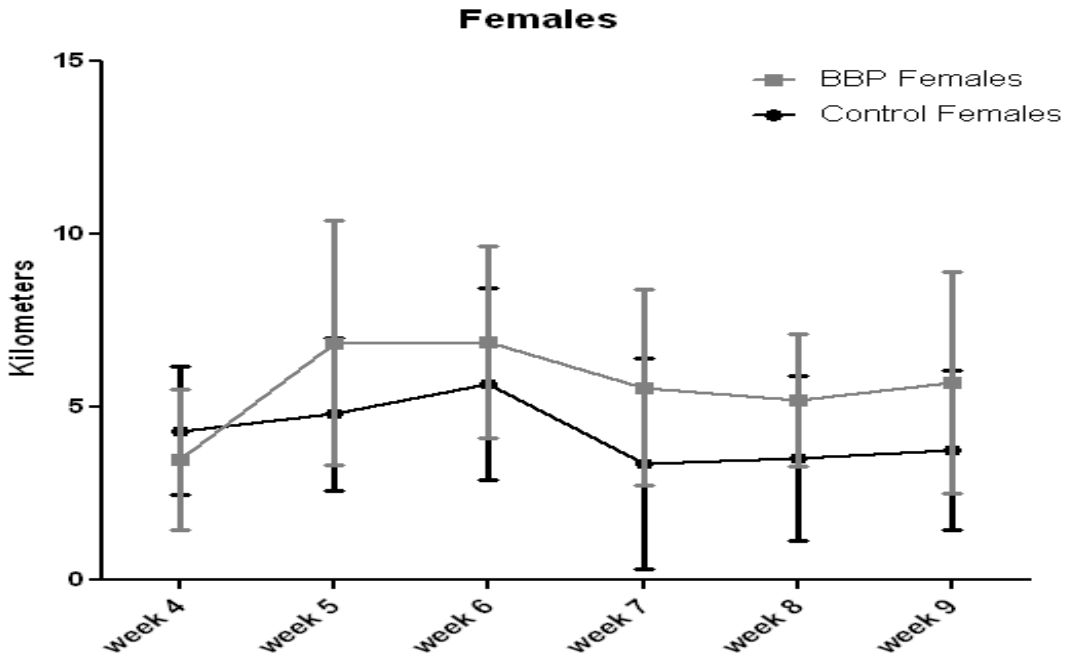

Figure 4: Activity data in females indicating no difference in running wheel distance throughout the study ((p=0.7235).

Chapter IV

DISCUSSION

This study's purpose was to determine if BBP administered prenatally would alter the formation of androgen receptors. Given the currently available results, BBP exposure did not affect androgen receptor formation in either male or female inbred mice. We had hypothesized that if androgen receptor function was inhibited by prenatal exposure to BBP, then this might result in an alteration of physical activity control. While not within the scope of the current project, our results were also supported by the observation of no alteration of physical activity levels in the subset of groups studied. However, we are hesitant to conclude that androgen receptor formation was not inhibited by BBP because of the small initial subset we have completed at this time. We anticipated that all data will be completed and analyzed within the next 30 days and will add to our story of how endocrine disruption affects androgen receptor formation and ultimately, physical activity. If our data continue to conclude that androgen receptor disruption was not achieved, other alternative methods of endocrine disruption must be considered to determine the role of androgen receptors in controlling physical activity.

Initially the dosage and administration of BBP must be analyzed. The dosage in the current literature suggests a 500 mg/kg concentration of BBP to be a harmful dosage in mice (11). Furthermore it has been shown that Bisphenol A (BPA), another endocrine disrupting compound, has been shown to be a marker of mammary cancer risk in mice given at a concentration of only 5 mg/kg given through an intraperitoneal injection into the pregnant mothers (12). This successful protocol lead us to give intraperitoneal injections of BBP. Compared to a paper by

Clewell, et al., which gave an oral givage, our protocol differed from the current BBP literature (11). This difference could explain the lack of substantial evidence for endocrine disruption.

If borne out in our completed dataset, the findings in this study question whether BBP actually exerts anti-androgenic effects. There has been controversy in the endocrine disruptor literature as to whether these substances have physiological-altering properties or even pose health risks. According to Witorsch, et al., BBP, even when present, is at too low a concentration to significantly pose any real adverse androgen effects in humans (13). This would suggest that although BBP present at high doses does pose a risk for developing androgen related disorders, the concentrations needed to produce these results are far from the actual concentrations encountered in daily living. Based upon our extremely limited analysis at this time BBP does not indicate a significant effect on the androgen receptors.

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