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Original Article

Postnatal wheel running mitigates endocrine disruption of mammary gland development in mice

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ABSTRACT — We investigated if access to a running wheel after *in utero* exposure to benzyl butyl phthalate (BBP) ameliorates the toxicological effects of BBP exposure. Our purpose was to determine if post-birth exercise after prenatal BBP exposure could reverse alterations in mammary gland development. Twenty-five female pups were exposed to 500 mg/kg of BBP on days 9-15 *in utero* and analyzed for mammary gland development and morphology. Mice either had access to a running wheel beginning at 8 weeks of age or were in a cage with a "locked" wheel to prevent running activity. Whole mount staining showed delayed mammary gland morphology development, regardless of wheel exposure in the treated groups. Additional histology staining revealed BBP exposed mice that were not allowed exercise, had larger ducts with multiple cell layers containing proliferative cells suggesting a favorable environment for tumor growth. In addition, there was a significant increase in progesterone status in the mouse mammary gland at 20 weeks but not 10 weeks, regardless of wheel exposure. BBP exposure led to abnormal mammary gland development in female mice and access to a running wheel helped ameliorate some, but not all, of the harmful effects due to BBP exposure at either 10 weeks or 20 weeks of age. Our results are significant because they indicate that exercise can reverse most of the BBP-initiated alterations in the mouse mammary gland, and physical activity has a positive impact on most developmental parameters in the mouse mammary gland.

Key words: Benzyl butyl phthalate, Mouse Development, Physical activity

INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) are both natural and man-made substances that can have a number of adverse health consequences on the human body. EDCs can affect the endocrine system and produce altered developmental, reproductive, neurological, and immune effects in humans and wildlife (Schug *et al*., 2011). Exposure to EDCs is more dangerous if the exposure occurs during "critical periods" of life (e.g., intrauterine, perinatal, or puberty periods) when organs are still developing and are more sensitive to hormonal disruption (Frye *et al*., 2012). However, little is known about the harmful effects of prenatal exposure to EDCs and physiological effects into adulthood (Rudel *et al*., 2011) even though it has been determined that exposure to certain ED in adulthood can alter physiology as well (Frye *et al*., 2012). Specifically, the mammary gland is sensitive to EDC exposure *in utero* because the mammary gland begins to form mammary buds during the embryonic stage of development (Brisken and Ataca). The placenta is not impenetrable to EDCs like it once was thought and it can-

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not fully protect the developing fetus from the toxicants (Newbold, 2011). In fact, the fetus can be more sensitive and more susceptible to environmental hazards than the adult (Newbold, 2011).

Since phthalates and other EDCs are found in cosmetics, perfumes, and beauty products, it has become an increasing concern that these common chemicals could affect female reproduction. Several human studies have found significant developmental effects from EDCs on the female reproductive tract. For example, the onset age of puberty in females has occurred earlier in life compared to past generations (Leonardi *et al*., 2017). Genetics (Navarro *et al*., 2004), nutritional status like obesity (Li *et al*., 2017), and the exposure to environmental chemicals have been postulated in causing this marked change (Choi and Yoo, 2013). The earlier onset of puberty has been liked to a slightly increased risk of breast cancer later in life (Bodicoat *et al*., 2014). Hormones such as estrogen, progesterone, and prolactin control female breast development and these hormones can become altered in individuals particularly when they enter puberty earlier (Wen *et al*., 2017).

The animal literature is not as clear on EDCs effect on puberty. Timing and dosing amount seem to make a vast difference on if female subjects reach puberty earlier or later. Research has shown in animal models that prenatal life exposure to tetrachlorodibenzo para dioxin (TCDD) can lead to delayed mammary gland development in rats (Brown *et al*., 1998). In fact, these females rats that demonstrated impaired mammary development also had a delay in onset of puberty and disruption to the estrous cycle indicating that hormone levels and production were severely impacted by TCDD prenatal treatment (Brown *et al*., 1998). In addition, researchers studied the effects of a phthalate mixture on the reproductive indices in mice (Zhou *et al*., 2017). They found female mice born to exposed mothers had significant increases in uterine weight, decreased anogenital distances, disrupted estrous cycles, and breeding complications (Zhou *et al*., 2017). These results indicate that exposure phthalates can have dire consequences in females, leading to reproduction disorders and perhaps even more complications as females age just from prenatal exposure.

We choose to study Benzyl Butyl Phthalate (BBP) because it is a well-characterized EDC found in a variety of personal care products women use daily (Hubinger and Havery, 2006; Koo and Lee, 2004). We have previously found that physical activity is controlled by sex hormones (Lightfoot, 2008) and that prenatal exposure to BBP leads to a decrease in physical activity in both male and female mice through a disruption of sex hormones (Schmitt *et al*., 2016). Specifically, our previous study found a decrease in anogenital distances in BBP-treated male offspring and a delayed vaginal opening indicating a later onset of puberty in female mice treat with BBP. The differences in puberty development between control mice and BBP-treated mice can be attributed to alterations in testosterone and estrogen concentrations in BBP-treated offspring. In addition, BBP-treated male and female offspring ran significantly less than their control counterparts indicating that BBP exposure *in utero* alters hormone production leading to differences to physical activity (Schmitt *et al*., 2016). Research has proven that there are a number of health benefits of exercise, including decreasing the risk of breast cancer in women (Wu *et al*., 2013) by reducing circulating levels of estradiol (Key *et al*., 2011). Since prenatal exposure to EDCs can lead to unfavorable breast changes (even cancer) in adulthood, the purpose of this study was to analyze mammary gland changes into adulthood from BBP prenatal exposure – and if any potential alternations from the BBP prenatal exposure – could be mitigated by voluntary physical activity during adulthood. No research has investigated whether *in utero* exposure to BBP combined with physical activity throughout adulthood can lead to favorable outcomes in breast health. Thus, our hypothesis is that free access to a running wheel will ameliorate the harmful effects on mammary gland development from *in utero* BBP exposure in 10- and 20-wk-old female mice.

MATERIALS AND METHODS

This protocol conformed to the standards of humane animal care and was approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2012-0274).

Animals

The experimental breeding and treatment protocol performed for this study can be found elsewhere (Schmitt *et al*., 2016). Briefly, twelve female breeder mice (C57BL/6J mice; Jackson Laboratory, Bar Harbor, ME, USA) were housed two to a cage with one male breeder (n = 6). Female mice were evaluated every 12 hr for the presence of a vaginal plug that indicated gestation day 0. Once gestational day 0 was determined, pregnant female mice were placed in individual cages and were administered a gavage treatment of either a control substance (100 µL of sesame oil) or 500 $mg \cdot kg^{-1} \cdot day^{-1}$ BBP in a vehicle of 100 µL of sesame oil on gestation days 9-16 when organ system development and testosterone production occurs (Nagao *et al*., 2000). While the BBP dosage we used was based

on previous studies (14, 15), it is estimated that fetuses are exposed to 1/100-1/1000 of the mother's dose of BBP (Moral, Sanucci-Pereira, *et al*., 2011). Thus, we estimate that the fetuses were exposed to ≈ 0.5-5 $mg \cdot kg^{-1} \cdot day^{-1}$ which places these exposure rates slightly above the US EPA safe dose for humans of 0.2 $mg \cdot kg^{-1} \cdot day^{-1}$ (United, States, and EPA, 2014). The resulting female pups (n = 25) were weaned at 3 weeks of age and then housed individually (with a freely turning running wheel or with a "locked" running wheel at 8 weeks) until terminated at the same time of day, either at 10 weeks (n = 16) or 20 (n = 9) weeks of age. At sacrifice, mouse mammary gland #4 was harvested due to its larger size and because this gland is the standard mammary tissue to study morphological or cellular changes in the mouse. Mammary tissues were then fixed in 4% paraformaldehyde overnight, washed with 1x PBS the following morning, and stored in 70% ethanol until future histological analysis.

Measurement of physical activity

Beginning at 8 weeks of age, physical activity measurements were determined on all animals in the "running" group (i.e., had a freely turning wheel) by measurement of daily distance ($km \bullet d^{-1}$), duration ($min \bullet d^{-1}$), and calculation of speed ($m \bullet min^{-1}$) of wheel running using our standard laboratory protocol (Lightfoot *et al*., 2004). In brief, running wheels with a 450 mm circumference and a 40 mm solid running surface were mounted to the cage tops of standard rat cages and were equipped with a computer (BC500, Sigma Sport, Batavia, IL, USA) to record running distance and duration. Running distance and duration data were collected on a daily basis in the morning, sensor alignment and wheel resistance checked and adjusted as needed, and an average daily running speed was calculated from the corresponding distance and duration measures. In addition, the mice with "locked wheels" in their cages, prevented exercise but enabled the same cage environment as the mice that had free-turning running wheels.

Carmine staining

We used Carmine staining (whole mounts) of the mammary gland to determine the branching of the ductwork in the mammary gland fat pad and to detail the formation of alveoli (Palmer *et al*., 2006). Tissues were fixed for two hours in 4% paraformaldehyde at 4°C then moved to 70% ethanol until staining. To begin staining, tissues were rinsed in PBS and stained in carmine alum solution (Carmine Sigma C-1022 and Aluminum Potassium Sulfate Sigma A-7167) overnight at room temperature. The following morning, tissues were washed in a series of ethanol dilutions, cleared in xylenes overnight, and stored in methyl salicylate at room temperature. Whole mount images were taken on the Zeiss SteREO Discovery.V12 (Carl Zeiss Microscopy GmbH, Jena, Germany). To analyze branching morphogenesis, whole mount mammary glands images were divided into six equal parts and branch junction points, and terminal ducts were counted in each relative position section.

Hematoxylin and eosin (H&E) and Masson's trichrome staining

The Histology Core Facility at the Texas A&M University College of Veterinary Medicine & Biomedical Sciences completed mammary gland tissue preparation and hematoxylin and eosin (H&E) staining, as well as Masson's Trichrome staining. H&E staining is the primary diagnostic technique used to evaluate morphology and is known as the "gold standard" for diagnosis of malignancies, and Masson's Trichrome staining is used to detect collagen fibers in the mammary gland. Specifically, ductal measurements taken from H&E stains were calculated by measuring the area of the full entire duct and also measuring the perimeter of the full duct. Next, measurements were taken of the lumen to determine area and perimeter measurements. The difference between the full duct and the lumen was calculated to give the area and perimeter (circumference) of the gland.

Immunohistochemistry (IHC) staining

For the remaining IHC staining, mammary glands from 10 and 20-week-old female mice were fixed in 4% paraformaldehyde solution. IHC staining was done to determine the differently expressed antigens in the mammary gland since H&E and Masson's Trichrome staining cannot easily identify the specific layers of the cells. Serial mammary sections were cut at 8 μm and used for IHC staining as described (Scribner *et al*., 2011). A standard IHC protocol was followed for all sections used in this study. Briefly, sections were deparafinized for 30 min at 60°C followed by 3-5 min washes in xylenes, 100% ETOH, 95% ETOH, 70% ETOH, and 1X PBS. Antigen retrieval was performed by boiling sections in 10 mM sodium citrate for five min. All sections were then washed in 1X PBS for five min, followed by a six min incubation in 3% hydrogen peroxide. Sections were then blocked for 60 min in PBS-T containing 10% horse serum. All sections were incubated overnight at 4°C in primary antibodies (Table 1) and washed in PBS-T for 10 min the following day. IHC proceeded with incubation for 60 min in secondary antibody (Table 1), a five min wash in PBS-T, a 30 min ABC (Vector Laboratories, Burlingame, CA, USA) incuba-

Table 1. Antibody Information for IHC.

Primary Antibodies

Target	Source	Cat. Num.	Species
Keratin14	BioLegend	905301	Rabbit
Smooth Muscle Actin	Abcam	Ab5694	Rabbit
Progesterone Receptor	NeoMarker	RM-9102-so	Rabbit
Ki67	NeoMarker	RM-9106-so	Rabbit

Secondary Antibody

Target	Source	Cat. Num.	Species
Goat anti-Rabbit	VectorLab	A11034	Goat

List of antibodies (primary and secondary) used in Immunohistochemistry analysis.

tion, and then an antibody-dependent timed DAB (Vector Laboratories) incubation. Sections were then counterstained in methyl-green and dehydrated with 95% ETOH, 100% ETOH, and xylenes, with coverslips mounted with Permount mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). Stained images were taken on the Zeiss Axioimager.Z1 at 10x or 40x (Carl Zeiss Microscopy GmbH).

To further investigate the differences between BBP treated mice and controls, we characterized the changes in the basal and epithelial cells of the mammary glands by staining for smooth muscle actin (Sato *et al*., 2014) and Keratin-14 (K14) to better observe the myoepithelial cell layer of the breast cells to gain a better understanding of the growth regulation, differentiation and morphogenesis of the neighboring cells (Sternlicht and Barsky, 1997). In addition, we stained for Ki67 to study the proliferation of the breast cells. Ki67 is a non-histone protein that is present at low levels during the quiescent state of the cell cycle, and Ki67 is increased in proliferating cells so this staining is done as a nuclear marker for cell proliferation. Since we have previously shown that female mice treated with BBP have disrupted hormonal profiles at 20 weeks (specifically higher levels of testosterone and lower levels of estrogen compared to controls; (Schmitt *et al*., 2016)) we next examined the progesterone receptor (PR) status of the mice at 10 and 20 weeks of age with a running or a locked wheel. Since PR status is under the control of and is a major target of estrogen, it is indicative of sex hormone functioning. Progesterone is required for side branching and coordination of ductal cell lobuloalveolar development (Brisken *et al*., 1998) and past work has shown that prenatal exposure to endocrine disruptors can inhibit hormone functioning and contribute to abnormal mammary gland development (Bern *et al*., 1987).

Statistical analysis

A one-way analysis of variance was performed for PR status between groups, as well as identifying differences between groups with the H&E staining. If the overall model indicated significance, then a Tukey's Post-Hoc analysis was performed to identify statistical differences between groups. ImageJ (open source image processing software, version 2.0.0-rc-68/1.52g) was used for analysis of ductal epithelium structures to specifically analyze the area and perimeter of mouse mammary ducts. Alpha levels were set to 0.05 *a priori* (JMP v.14.1.0, SAS, Inc. Cary, NC, USA).

RESULTS

Whole mount staining

Whole mount analysis of glands from the 10-week and 20-week old BBP and control mice that had access to running wheels showed morphological differences in BBP exposed mammary glands relative to control glands at 10-weeks old (Fig. 1, A-B). BBP exposed mammary glands visually appear to have fewer bifurcations 10-weeks (Fig. 1, A); however, BBP mice showed no morphological differences from control mice by 20-weeks (Fig. 1, B).

Histological staining of the mammary gland

Overall, the histological staining of the mouse mammary gland indicated that female mice exposed to BBP *in utero* and that did not have access to a running wheel had mammary glands that were negatively impacted on a cellular level compared to mice that ran on a wheel. Mammary glands that display negative changes in cellular structure have a greater chance of developing pre-cancer lesions and if untreated, these lesions could turn into cancer. The BBP treated mice without access to running

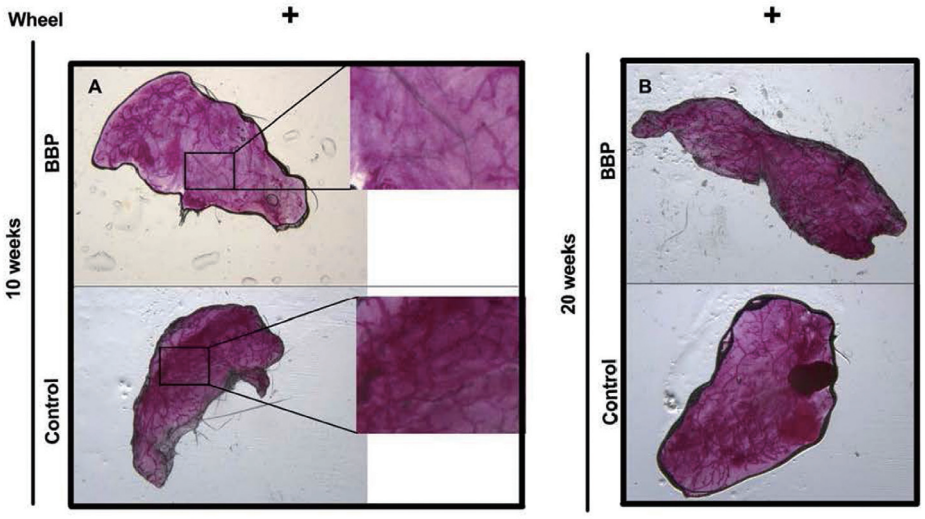

Fig. 1. Whole mount staining. Whole mount staining of mammary glands from 10-week-old mice that had access to a running wheel. In BBP mice at 10 weeks (A), there is a delay in the development of the mammary gland. By week 20 (B), the BBP running animals catch up developmentally to the control counter parts.

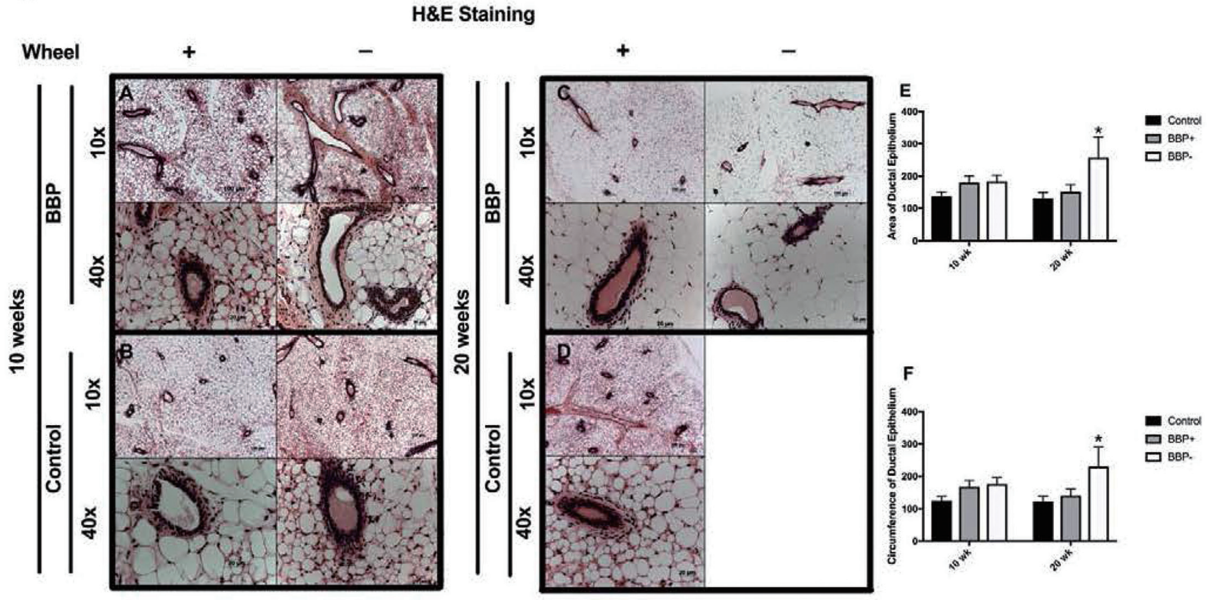

Fig. 2. H&E Staining. H&E staining of Control and BBP treated mice with and without access to a running wheel at 10 and 20-weeks. BBP treated mice without access to a running wheel have larger ducts with multiple cell layers not found in the control mice at 20-weeks (E, F). This is evident at week 10 (A, B, E, F), but more pronounced and statistically significant by week 20 (C, D, E, F) in BBP running and locked wheel mice. Glands shown in the 10x and 40x magnification group have a scale bars of 100μm and 20μm, respectively. Data is represented mean with SD error bars. *($p < 0.05$)

wheels had larger ducts with multiple cell layers around the ducts (Fig. 2, H&E staining) not found in the control mice. This pattern was visibly evident at 10-weeks of age (Fig. 2, A-B) but was more pronounced and statistically significant by week 20 (Fig. 2, C-D, E-F) in BBP exposed mice with locked running wheels. BBP mice

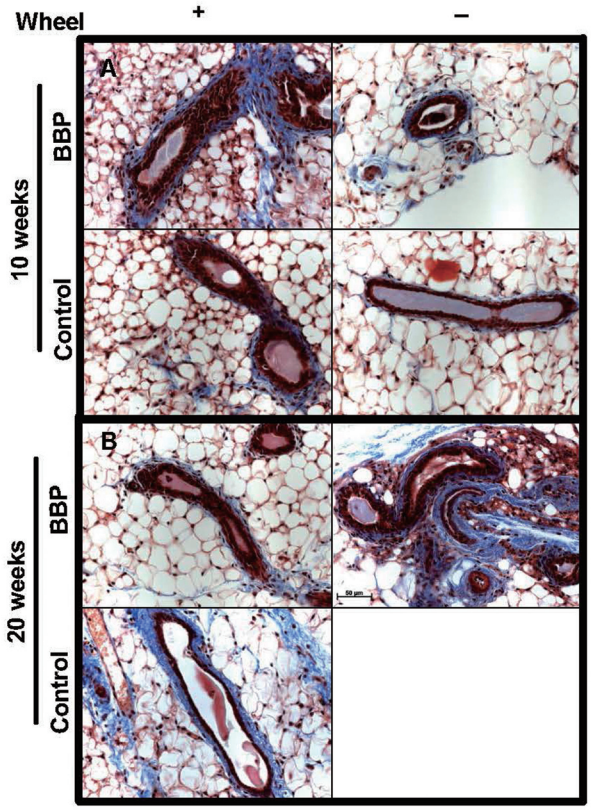

Fig. 3. Masson's trichrome staining. Mason's trichrome staining of Control and BBP treated mice with and without access to a running wheel at 10 and 20-weeks. The blue staining represents collagen fibers, the red staining indicates different cell layers, and the dark brown staining is of cell nuclei. Mice that were exposed to BBP in utero and did not have access to a running wheel showed multiple cell layers with proliferative cells infiltrating surrounding healthy tissue by week 20 (B). Images were taken at 40x and have scale bars of 50 μm.

with locked wheels at 20-weeks of age had larger ducts ($p = 0.0322$) compared to age-matched controls (Fig. 2, E). Next, we measured the circumference of the ductal epithelium and found a significantly larger circumference of epithelial ducts in 20-week BBP mice with a locked wheel ($p = 0.0475$) compared to age-matched controls (Fig. 2, F). Mice that were exposed to BBP in utero and did not have access to a running wheel showed multiple cell layers with proliferative cells infiltrating surrounding healthy tissue by week 20 (Fig. 3, B). Ki67 staining also confirmed that BBP treated mice on locked wheels had larger ducts with multiple cell layers containing more proliferative cells compared to control mice at 10 and 20 weeks (Fig. 4, E-F). These findings are significant because they show that exercise into adulthood has a positive effect on mammary gland development even though these mice were exposed to a harmful endocrine disruptor *in utero*.

In a few cases, IHC staining indicated that BBP treated mice regardless of exercise status, showed an increase in smooth muscle actin (Sato *et al*.) at 10 weeks (Fig. 4, A) and also at 20 weeks (Fig. 4, B) compared to control mice. SMA antibody recognizes the alpha-smooth muscle isoform of actin and stains the myoepithelial cells in breast tissue. Further, we found that the luminal epithelial cell layer contained more K14 positive cells in BBP mice on running wheels and locked wheels compared to the control mice at 10 and 20 weeks (Fig. 4, C-D).

Progesterone receptor (PR) status

BBP *in utero* exposure led to increased PR positive cells (Fig. 5, E) in mice that were 20-weeks old regardless of running wheel accessibility. However, there were no significant changes in PR cell status at 10-week of age (Fig. 5, E) in mice that had free access to a running wheel ($p = 0.2250$) and in mice that were not allowed to run on a locked wheel ($p = 0.3211$) compared to an age-matched control on a wheel. These results suggest that BBP exposure has direct impact on sex hormone function at 20-weeks, and not at 10-weeks of age, regardless of wheel exposure.

DISCUSSION

Participation in daily exercise has been shown to reduce one's lifetime risk of developing and preventing a reoccurrence of breast cancer through a reduction in obesity (Picon-Ruiz *et al*., 2017; Sturgeon *et al*., 2016), but no studies have directly examined the combination of phthalate exposure *in utero* and physical activity in adulthood on mammary gland development. The purpose of this study was to determine if access to daily exercise altered the effect of *in utero* BBP exposure on mammary gland development and morphology in mice.

BBP is classified a reproductive toxicant with studies suggesting that reproductive development in females may be adversely impacted by phthalate exposure during *in utero* development (Watkins *et al*., 2017). Normal female mammary gland development in mice requires a well-coordinated series of developmental events starting with budding and branching *in utero* with only a rudimentary ductal system present at birth. During puberty the production of hormones (estrogen and progesterone) cause growth of mammary terminal end bud epithelial cells.

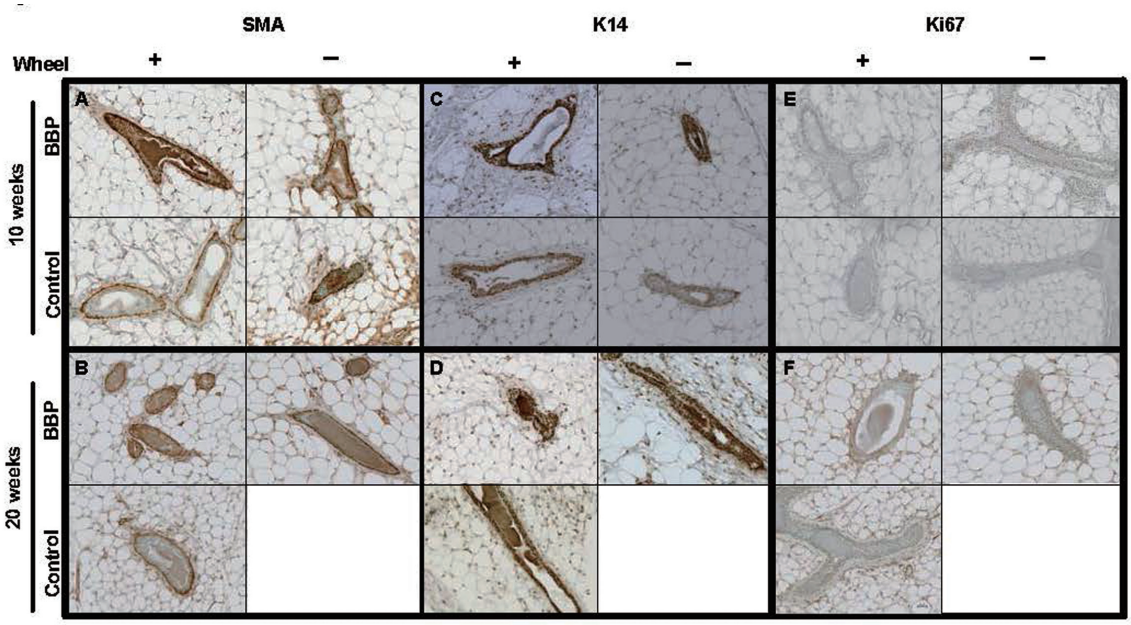

Fig. 4. K14 & SMA staining and Ki67 as a marker for proliferation. K14, SMA, and Ki67 staining of Control and BBP treated mice with and without access to a running wheel at 10 and 20-weeks. The luminal epithelial cell layer shows contained K14 positive cells in BBP mice on running wheels and locked wheels compared to the control mice that had a "normal" cell layer profile at both 10 and 20 weeks (C, D). In addition, there is an overexpression of Ki67 cells in mice exposed to BBP in the 10 week and 20-week group (E, F). BBP mice on locked wheels had higher expression of Ki67 positive cells compared to BBP mice on running wheels (E, F). Images were taken at 40x magnification and have scale bars of 20 μm.

These cells continue to grow and more side branching occurs to prepare for pregnancy and eventually lactation and involution (Brisken and Ataca, 2015). In a rat study similar to ours, researchers found that *in utero* BBP exposure of 500 mg/kg resulted in a decreased number of terminal end buds in treated animals (Moral, Santucci-Pereira *et al*., 2011). In the current study, the decreased side branching and terminal end buds in BBP treated mice at 10 weeks but not 20 weeks (Fig. 1) suggests that morphological development is delayed compared with BBP exposure, but not permanently. This conclusion is supported by our previous research that showed female mice had a significant delay in vaginal opening with prenatal exposure to BBP compared to controls (Schmitt *et al*., 2016). In addition, the increase in cell proliferation in BBP exposed mice, as well as differences in SMA, K14, and PR markers (Fig. 4, Fig. 5) suggests mammary gland development is altered by prenatal BBP exposure. Our results indicate that the mice exposed prenatally to BBP have the potential for tumor development. Although the treated BBP mice did not develop breast tumors in our study, cell proliferation is increased in tumors (Gupta and Massague, 2006) suggesting that prenatal exposure to an endocrine disruptor could potentially cause mammary gland impairments in adulthood. The literature has also indicated that larger ducts in the mouse mammary gland could signify a greater chance for proliferative potential (Fernandez-Gonzalez *et al*., 2009), and our current study found larger ducts in BBP-treated mice at 20-weeks that were on locked wheels (Fig. 2, E-F). Exercise may not fully offset the effects of BBP exposure on the delay and alteration of mammary gland development because we observed that PR functioning was altered in mice at 20 weeks regardless of daily activity (Fig. 5, E). The dysregulation of hormone function is not surprising given our previous findings that *in utero* BBP exposure led to changes in estradiol levels at week 20 with a decrease in testosterone, leading to an overall reduction in physical activity in female mice (Schmitt *et al*., 2016) (Fig. 6).

The American College of Sports Medicine suggests that adults should engage in at least 150 minutes of moderate-intensity exercise per week (Haskell *et al*., 2007). The recommendations can be broken down to 30-60 min of moderate-intensity exercise over five days or 20-60 min of vigorous-intensity exercise three times per week (Haskell *et al*., 2007). Specifically, after a diagnosis of breast cancer, studies have shown exercise to be a beneficial part of treatment during chemotherapy

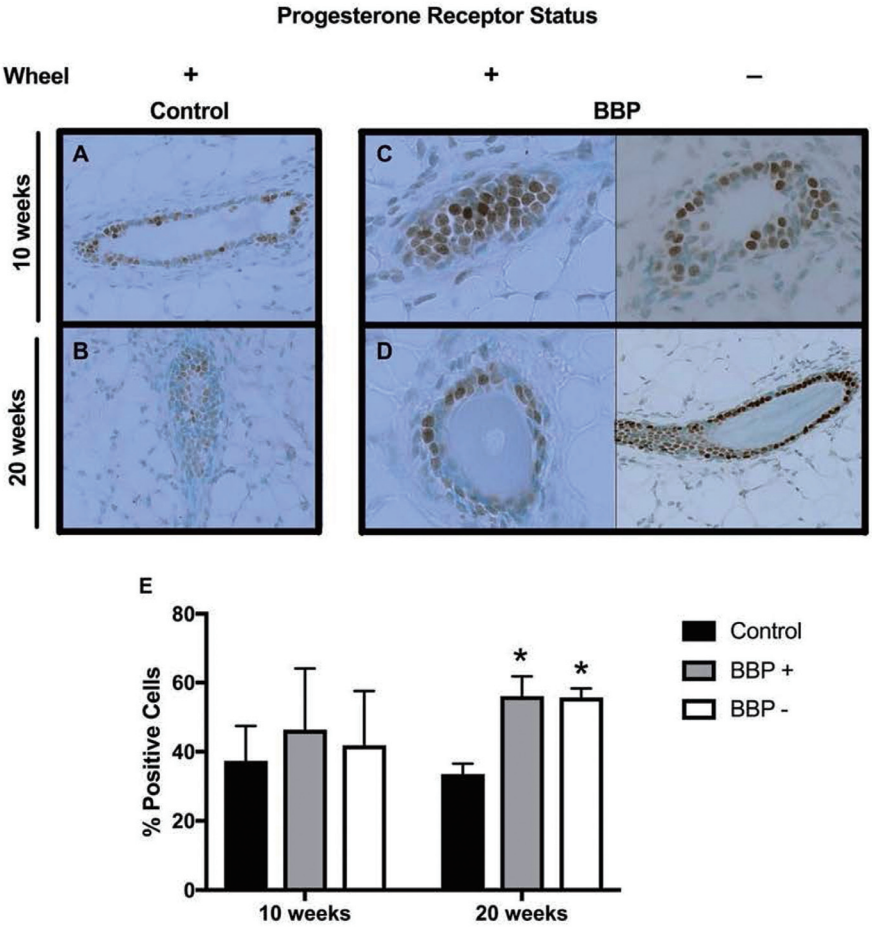

Fig. 5. Progesterone receptor (PR) status. PR staining of Control and BBP treated mice with and without access to a running wheel at 10 and 20-weeks. BBP in utero exposure leads to increase in PR positive cells (not at 10 weeks but 20 weeks) indicating BBP exposure has direct impact on sex hormone function, regardless of wheel exposure (A-E). Data is represented mean with SD error bars. Images were taken at 40x magnification and have scale bars of 20 μm. *($p < 0.05$)

(Courneya *et al*., 2014), as well as a beneficial treatment to prevent reoccurrence (Magne *et al*., 2011). Mice born after *in utero* exposure to BBP do not voluntarily run as much as their control counterparts (Schmitt *et al*., 2016), so even though exercise may be able to delay or prevent breast cancer development, mice exposed to BBP may not be able to overcome the early life exposure that has impacted mammary gland development even with exercise in adulthood. Our results support this hypothesis because exercise exposure only altered some histological parameters in the mouse mammary gland. For instance, mice exposed to BBP *in utero* allowed to run freely in adulthood demonstrated -for the most part- normal mammary gland biology (i.e. smaller mammary ducts only containing a single layer of cells). Daily running in these animals prevented the mammary ducts from developing potential harmful lesions. The BBP-treated mice that were allowed to run freely on a running wheel during the study did not have an increase in ductal area or circumference (Fig. 2, E-F), indicating that perhaps, wheel running (even after exposure to BBP *in utero*) can act as a protective mechanism for mouse mammary ducts to prevent cell proliferation.

Limitations and conclusions

A few limitations are apparent with this study. First, due to a limited number of animals, as well as, missing a "no wheel" 20-week control group, we were unable to perform statistics for some of the given mammary gland staining. Even though this is a limitation, we performed as

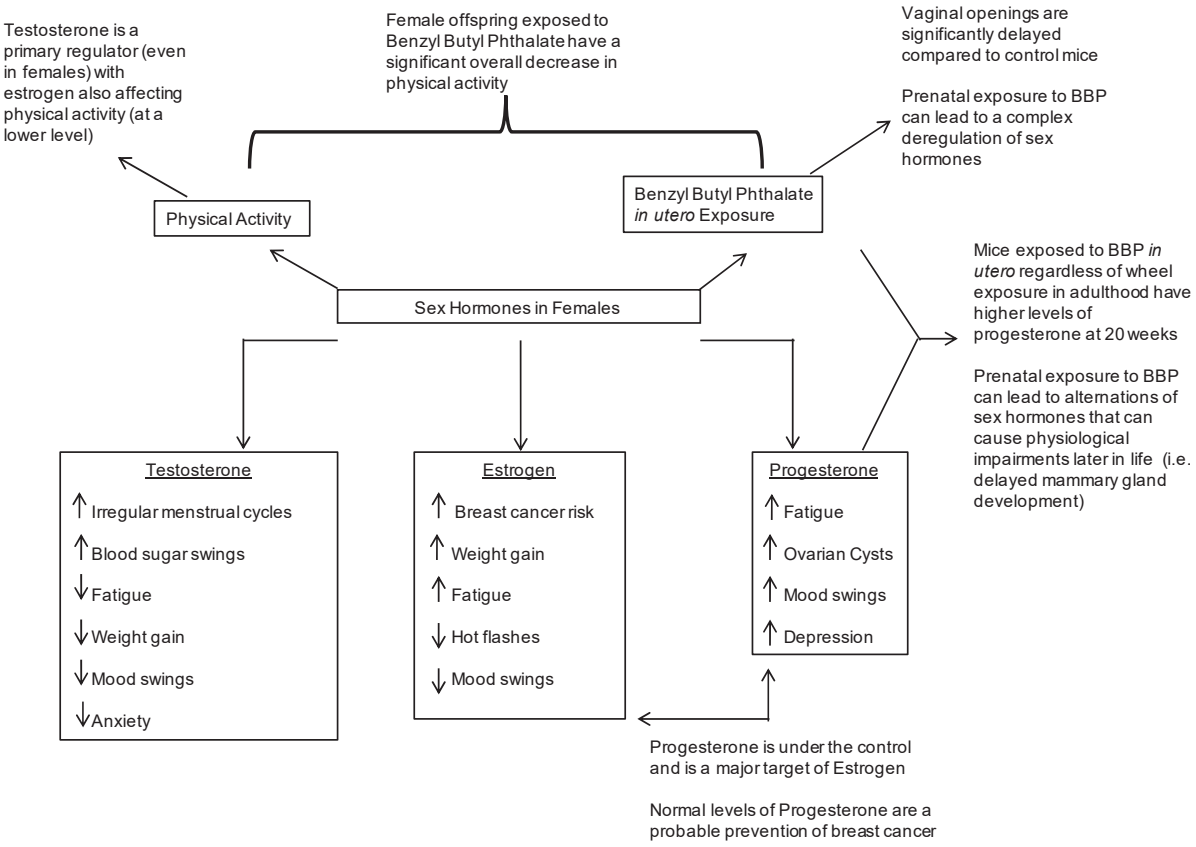

Fig. 6. Overall schematic of sex hormones in females. This figure represents the control of sex hormones in females. There are 3 sex hormones in females: estrogen, progesterone, and testosterone. Each of these sex hormones plays a vital role in coordinating hormonal functions within the human body, and these hormones are major players in mammary gland development. In the boxes labeled testosterone, estrogen, and progesterone, the up arrows indicate a certain response if that hormone is elevated, and the down arrows indicate a certain repose if that hormone is decreased.

much IHC as we could to show as much of the developmental mammary gland story as possible throughout the 20-week life span. Next, mice were not staged for estrous which is a possible explanation for some of the visual differences seen in some of the markers observed – particularly, Ki67. Despite these limitations, the current study shows for the first time that mice exposed *in utero* to BBP and have access to a running wheel in adulthood, mitigate some of the mammary gland alternations occurring as a result of phthalate exposure. While our previous work has shown that *in utero* BBP exposure decreases daily physical activity (Schmitt *et al*., 2016) the current results are significant because they indicate that even though exercise did not reverse all of the BBP-initiated alterations in the gland, physical activity did have a positive impact on most developmental parameters in the mouse mammary gland. This finding concludes that some exercise is better than no exercise in reversing potential alterations of the mammary gland from endocrine disruption prenatally. As we work to continually understand how sex hormones play a role in physical activity (Fig. 6) and how exercise can lower cancer risk, our data suggest that effects of environmental toxicants on the "physical activity/cancer" axis play a large role in the eventual outcome. Thus, more research is needed to discern the mode, duration, and intensity of exercise needed to reverse potential alterations in mammary gland development due to prenatal exposure to an endocrine disruptor. Prenatal exposure to BBP can lead to alternations of sex hormones that can cause physiological impairments later in life, and these changes in hormone profiles could explain why mice exposed to BBP *in utero* that had access to a running wheel still had some anatomical alterations in mammary gland development.

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Conflict of interest---- The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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