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In Utero Exposure to Benzo [a] pyrene Results in Ovarian Follicular Depletion in F1 Muta™ mouse

THESIS

Submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

In Environmental Health Sciences

By

Sanjiwani Meharda, MD, MBA

Thesis Committee:
Ulrike Luderer, MD, MPH, PhD
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2016

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ABSTRACT OF THE THESIS

By

Sanjiwani Meharda

Master of Science in Environmental Health Science

University of California, Irvine,2016

Professor Ulrike Luderer, Chair

In contrast to gametogenesis in males, oocytes in females are not continually replenished during reproductive life. Exposure to ovotoxicants during or after embryogenesis can lead to irreversible ovarian dysgenesis or premature ovarian failure (POF).

Benzo [a] pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) and a component of air pollution. PAHs are formed as a result of incomplete combustion of organic materials and are ubiquitous environmental contaminants. Found in cigarette smoke, polycyclic aromatic hydrocarbons are "toxic to and destructive to oocytes in several animal test systems." Men and women who smoke have decreased fertility.

Our study for the first time uses the *lacZ* transgenic Muta™Mouse, mouse model to study female reproductive consequences of prenatal exposure to BaP Pregnant transgenic MutaMouse were treated with 0, 10, 20 or 40 mg/kg/d BaP orally from gestational day 7 through 16, which represents the major period of organogenesis. The F1 female offspring were euthanized at 10 wk. of age and

ovaries were processed for differential ovarian follicle counting.

Transplacental BaP exposure caused very significant dose-related decreases in primordial, primary and secondary follicles in the ovaries of F1 MutaMice. There was prominent ovarian tissue destruction at the higher doses of BaP exposure. Our results show that maternal exposure to BaP during gestation is highly damaging to the developing ovaries of the F1 female offspring in Muta mice as it is in other mouse strains that have been studied. Therefore, this model is an excellent model not only for observing the mutation effects but also for showing the reproductive effects.

Our findings in the mouse are relevant to humans, as ovarian development in humans and mice is very similar. Increasing evidence regarding PAH exposure and its harmful effects leads us to believe that additional regulations to reduce PAH exposure are needed at the government level. In addition, campaigns to increase awareness among the general population about PAHs are needed, as "Prevention is the key".

1.0 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one of the most ubiquitous groups of environmental toxicants (NHANES, 2009). They are formed by incomplete combustion of coal, oil, gas, wood, garbage or other organic substances. PAHs occur in nature in the form of combustion products from wild fires, crude oils, coal tar or creosote (ATSDR, 1995). They usually occur as complex mixtures rather than single compounds. Most PAHs to which humans are exposed are anthropogenic in origin, e.g. coke, carbon black, petroleum, home heating, and electric power generation. Medicines, plastics, dyes, pesticides and roofing tar are some examples of manufactured PAHs.

1.1 Chemical Nature

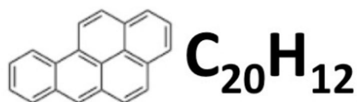
As pure chemicals PAHs are colorless, white or pale yellow-green solids. They have a pleasant faint odor. Chemically PAHs are fused benzene rings containing carbon and hydrogen atoms arranged in linear, angular or clustered fashion (Gachanja, 2009). When nitrogen, sulfur and oxygen atoms are substituted in the benzene rings, they are classified as heterocyclic aromatic hydrocarbons (cdc.atsdr.gov, 2013).

There are more than 100 PAHs prevalent in nature, but mainly 17 PAHs are discussed in the ATSDR Toxicological Profile. These PAHS are the most commonly found PAHs in Superfund designated and other polluted sites. A lot of studies have

been done on these PAHs regarding their chemical structure, toxicokinetics, and possible toxic effects on animals and humans.

Since there are many similarities in their toxicological profiles, most of the individual chemicals have been classified under one single group called as PAHs. We have chosen to focus on Benzo[a]pyrene(BaP), because it is a well characterized human carcinogen and has been extensively studied.

1.2 BENZO (A) PYRENE (BaP)



Benzo (a) pyrene (BaP) is a crystalline aromatic hydrocarbon, consisting of five fused benzene rings. It is one of the most extensively studied PAHs, and can be considered the prototype for most of the PAHs. BaP is listed as group 1 carcinogen by IARC (IARC, 2015). BaP can contaminate soils when dispersed from hazardous waste sites; it can contaminate water through industrial waste treatment plants, air through exhaust from automobiles or wood burning, or food through grilling or smoking (ATSDR, 1995).

1.3 Exposure Routes

BaP enters into the body via inhalation of smoke or contaminated air through lungs. BaP can be absorbed via gastrointestinal tract by swallowing contaminated food or water (Bostrom et al, 2002). Dermal absorption can also be a significant route of exposure especially in the occupational settings.

Indoor activities, such as use of gas for cooking or heating and cigarette smoking, are significant sources of BaP in the indoor environment; however, ingestion of contaminated food items poses a greater health threat to the US population as compared to inhalation and dermal exposure. In a study done by Frey and coworkers (Frey et al, 1991), a multimedia transport model was used to evaluate the distribution of BaP in the environment. Their results showed that the main source of human exposure was the food chain, accounting to 97 % of total daily intake of BaP. They also suggested that the average daily intake of BaP by general population is about 2.2 mcg/day. More recent studies provide even better estimates "Among the study subjects, the range and magnitude of dietary exposures (2 to 500 ng/d) were much greater than for inhalation (10 to 50 ng/d). Nevertheless, there were ample individual cases where inhalation of BaP was the predominant exposure route." (Waldman et al, 1991).

Concentrations of PAHs were measured in the busy streets of Copenhagen and an adjacent park. The concentrations were found to be maximum in the street followed by suburbs and minimum in the open land. The traffic contribution of PAHs to the street air was 90 % on the weekdays and 60 % on the weekends (Torben et al,

1996). Therefore, in urban polluted cities, inhalational exposure to PAHs may be the main exposure route.

The ingested or inhaled PAHs are only partially absorbed by the body; the absorbed fraction is referred to as "internal dose". This dose is a better measurement of the toxicity as compared to the amount exposed, termed as "External dose". (Hrudey et al, 1996; Paustenbach, 2000). About 30% of ingested BaP is absorbed via the gut (Cavret et al, 2003). BaP is lipophilic, having affinity to fatty tissues. Therefore, the main storage sites in human body are liver, kidneys, and adipose, but small amounts may be stored in spleen or adrenals. BaP has been shown to have great affinity to ovarian tissues (Bostrom et al, 2002). It crosses the placental barrier, thereby, posing risk to both the mother and developing fetus.

1.4 Metabolism

Metabolism of BaP is a complex process creating toxic metabolites, which are more harmful than the parent compound. The liver is the principal organ of metabolism for BaP. Other tissues including the ovary are also capable of metabolizing BaP into intermediate toxic metabolites (Wall et al, 1991). The major cytochrome P450s involved in BaP metabolism are CYP1A1, CYP1A2 and CYP1B1 (Eling et al, 1986; Shimada, 2006). Cytochrome P450s are activated by binding of BaP to the aryl hydrocarbon-receptor (AhR) nuclear complex, which in turn leads to the changes in gene transcription of CYPs and phase-II enzymes. The AhR regulates the transcription of a series of genes including CYP1A1, CYP1A2, and GST A1 (glutathione S-transferase A1) along with other several genes. All these genes are activated by AhR-ligands, including BaP, via the AhR-mediated aromatic

hydrocarbon response element. The AhR also plays a role in the response to oxidative stress in cell-cycle regulation and apoptosis. In addition, the CYP1A1/ CYP1A2-mediated metabolism generates oxidative stress (Nebert et al, 2000). Mice lacking the AhR receptor are refractory to BaP-induced tumorigenesis (Shimizu et al, 2000).

Most of the metabolic pathways reflect genotoxic mechanisms, as they involve alterations to DNA. BaP is pleotropic and has the ability to affect many cell- and organ-based systems. There are probably many modes of actions operating to different extents, which include mechanisms that involve AhR, oxidative stress, immunotoxicity and epigenetic events (IARC 2012). The current understanding of mechanisms underlying BaP-induced toxicity in experimental animals is mainly based on two pathways: those of the diol epoxides and the radical cations. Each provides a different explanation for the effects observed in experimental animals in specific tissues. (Xue et al, 2005)

- **Bay region dihydrodiol epoxides pathway**

The bay region dihydrodiol epoxides pathway involves three enzyme-mediated reactions. The first is oxidation of a double bond to unstable arene oxides; CYP450 enzymes catalyze this. Second, arene oxides are hydrolyzed to trans dihydrodiols, catalyzed by microsomal epoxide hydrolase. Finally, a second CYP-catalyzed oxidation at the double bond adjacent to the diol function occurs to generate a vicinal diol-epoxide. The diol epoxides are electrophilic and capable of binding to DNA, forming ultimate carcinogens (Baird & Ralston, 1997; Harvey,

1991). Diol epoxides react with DNA, mainly with the purines, forming DNA adducts BaP-7, 8-diol-9, 10-epoxide, or BPDE, which is considered to be an ultimate, DNA-reactive, metabolite of BaP (Osborne & Crosby et al, 1987).

- **Radical cation pathway**

Radical cations are formed when one electron is removed from the electron system of the molecule via one electron oxidation. Radical cations are electrophilic in nature; they interact with nucleophilic centers in cellular macromolecules. The formation of radical cations in metabolic oxidation process is catalyzed by CYP450 peroxidase (Cavalieri & Rogan et al, 1976, 1992, 1995). The radical-cation mechanism for BaP has been studied exclusively in connection with mouse skin tumorigenesis (Cavalieri & Rogan, 1995). One-electron oxidation of BaP by CYPs or peroxidases creates a radical cation localized on carbon 6, as a consequence of the ionization potential and geometric configuration. This radical cation forms covalent adducts with guanine and adenine at C8 and N7 sites. The radical cation adducts of BaP induce mutations in mouse skin, causing papillomas (Cavalieri & Rogan, 1995; IARC, 2012)

Phase 1 metabolites undergo conjugation with glutathione, sulfates, or glucuronic acid. This process occurs in the cytosol. The phase II metabolites like 4,5-diol glucoronide; 9,10-diol glucoronide; 3(OH) glucoronide; 3(OH) sulfate; 9(OH) sulfate; 7,8- diol GSH (Glutathione); and 9,10-diol GSH are more hydrophilic and hence amenable to elimination through excretion via bile, urine or feces (Ramesh et al, 2001).

Metabolites like phenols, quinones and dihydrodiols can be identified in body tissues and excreta. These metabolites are usually isolated as glucoronide or sulfate ester conjugates in the excreta but can also include glutathione conjugates formed from quinones or intermediary epoxides. The primary route of metabolite elimination is in the feces, particularly following exposure by the inhalation route. To a lesser degree BaP metabolites are eliminated via urine. Overall, BaP is eliminated quickly with a biological half-life of several hours (www.epa.gov/iris). The prerequisite for conversion of BaP into the toxic metabolites like diol epoxide is the presence of CYPs and epoxide hydrolase. Factors like distribution to the target tissue, solubility, intracellular location, and ability to activate mixed function oxidases, are all very important factors determining the fate of BaP exposure.

1.5 Benzo (A) pyrene (BaP) metabolism in Ovary

Various studies have proven BaP to be an ovotoxicant; however the toxicity is mainly by intermediate compounds rather than directly by the parent compound. Ovarian enzymes mainly involved in biotransformation are CYP1A1 and epoxide hydrolase (Bengtsson et al, 1988). BaP is distributed in the ovary, where the ovarian enzymes convert the parent compound to toxic metabolites (Mattison et al, 1983). Studies have shown increased concentration of DNA adducts in the ovary as compared to liver, suggesting that this organ is more vulnerable to damage by BaP (Ramesh et al, 2010). "A sustained BaP metabolite load was registered in the ovaries even after a single acute exposure to BaP" (Ramesh et al, 2010). Suggesting that ovary is a sensitive organ for toxicity.

Mattison et al, in their experiment in 1989, showed that there were pathways of direct toxicity to ovary. Intra ovarian injection of BaP or its metabolites, 7, 8 oxide, dihydrodiol (DHD), and diol epoxide, were administered to 3 different strains of mice. (DBA/2N (D2), C57BL/6N (B6), and (DBA/2N × C57BL/6N)). 6-8 week old mice were treated with injection of 10-µg compound mixed with 1 µg /L DMSO in the right ovary, the left ovaries were untreated. Two weeks following treatment both ovaries were removed. The results showed that the treated ovaries had decrease in weight and volume. There was a decrease in small, growing, and antral follicles in all the strains of mice. The untreated ovary was hypertrophied. The results suggest that BaP metabolites are potent ovarian toxicants. These data also suggest that the ovary is fully capable of metabolizing a xenobiotic-like BaP into reactive products capable of producing oocyte destruction.

Direct toxicity to the ovarian tissue is can also be a result of formation of ROS. Inhibition of BaP metabolism by supplementation of alpha naphthoflavone (Ah receptor antagonist) prevented oocyte destruction; this substantiates the theory that BaP must undergo bio activation to arene oxides in order to exert the ovotoxic effect (Mattisson et al, 1983).

1.6 Ovarian Development

Ovary is the main reproductive organ in females, along with fallopian tubes and uterus. The main roles of ovary are oogenesis and production of sex hormones (Peluso, 1992). Development of a normal ovary during fetal life is mandatory for normal folliculogenesis in adult life. The development of the ovary is dependent on

the presence of female specific genes and absence of sex determining region on the Y chromosome (SRY).

During embryogenesis the primordial germ cells (PGC/ also termed as oogonia) migrate to the genital ridge. The oogonia proliferate to establish a large pool of germ cells. During mitotic phase germ cells proliferate to form nests, which are a group of oogonia surrounded by somatic cells. The latter are the future pregranulosa cells of primordial follicles. The group of oogonia undergoes synchronized mitosis thereby forming clusters of gametes (Sarraj et al, 2012). The oogonia enter meiosis around gestational day 13.5 in mice; while in humans, oocytes are arrested in the diplotene phase of meiosis from 2-7 months of fetal development (Baker, 1963; Peters et al, 1976). The epigenetic regulation of gene expression occurs at the time of organogenesis thereby insults during this time may lead to heritable changes in gene function.

The mammalian ovary has a fixed number of primordial follicles with oocytes arrested in prophase 1 of meiosis (Pepling et al, 1999). Once this pool is established, follicles gradually and continually leave the resting pool to begin further growth. The follicles during the growth may meet two fates, either ovulation or atresia (Fortune et al, 1994).

1.7 Ovarian function in the adult ovary

The outer portion of the ovary containing most of the primordial and primary follicles is called cortex. The inner portion, consisting of larger follicles and stroma, is called the medulla. The cycling ovary contains ovarian follicles at different stages of development, depending on the ovulation and species. The process by which the

immature follicles develop into preovulatory follicles is called folliculogenesis (Oktem & Urman et al, 2010). This process is very dynamic and is tightly controlled by the gonadotropin hormones FSH (follicle stimulating hormone) and LH (Luteinizing hormone) (Mugaffin et al, 2005). Four major regulatory events are involved in folliculogenesis, 1) Recruitment, 2) Pre-antral follicle development, 3) Selection and 4) Atresia.

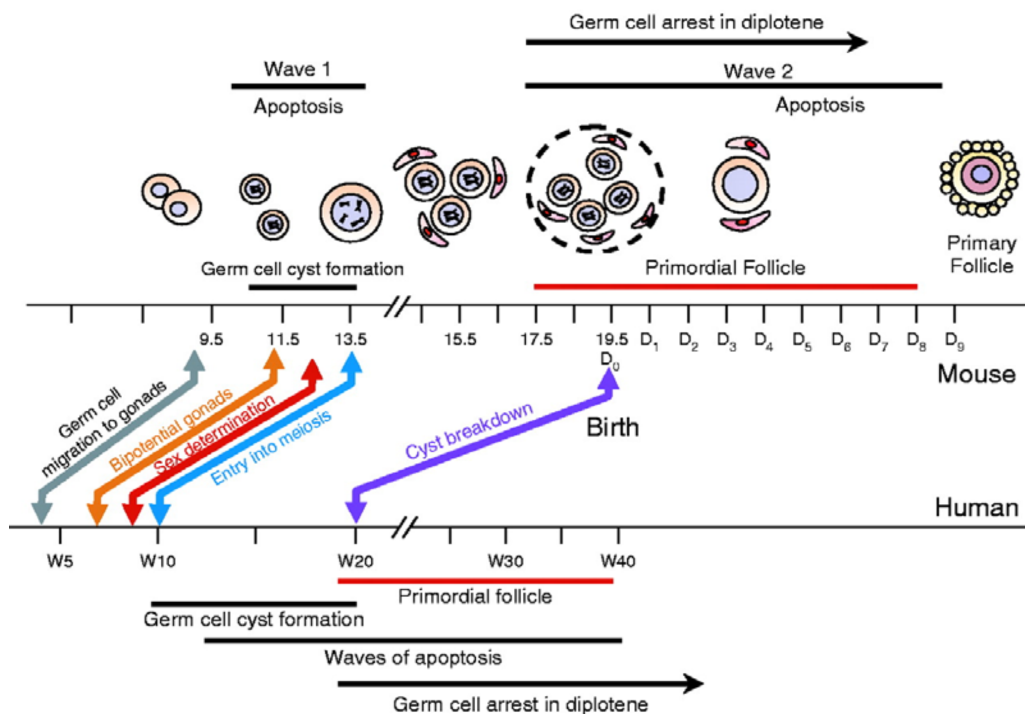


Figure: 1 - Mouse and human timelines for major processes taking place during fetal development

(Mai A Sarraj, and Ann E Drummond Reproduction 2012; 143:151-163)
(As reviewed in Pepling (2006) and Hartshorne et al. (2009)). In W (weeks post coitus). D0 is the day of birth in the mouse

Primordial follicles consist of a single oocyte arrested in the diplotene stage of the first meiotic prophase, having a single layer of squamous cells around it. These follicles exist in finite number in the ovary, constituting the ovarian reserve, and in

the process of recruitment are triggered to enter into the growing follicle pool (Fortune et al, 1994). As the process continues, the granulosa cells gradually change into cuboidal shape, forming multiple layers around the oocyte. These are now classified as primary (single layer of cuboidal granulosa cells) and secondary follicles (multiple layers). The transition from the primary to secondary follicle (also called as pre-antral follicle) is the accumulation of 2-10 layers of cuboidal or columnar epithelial cells (Erickson et al, 1985). At the same time when the oocyte is differentiating with significant RNA synthesis, a number of oocyte genes are turned on, especially the genes encoding zona pellucida proteins. This is important for signaling the formation of thick layer of extra cellular matrix around the oocyte called the zona pellucida (Bachvarova et al, 1985). Simultaneously the theca cells differentiate from the stromal cells outside the basement membrane that forms the boundary of the granulosa cells. During this process angiogenesis is also taking place within the theca cell layer, providing oxygen and nutrients to developing oocyte.

When the pre-antral follicle completes the secondary stage it contains an oocyte, surrounded by a proteinaceous structure called the zona pellucida, multilayered granulosa cells, the basal lamina, and theca interna and externa cell layers. During the last phase a cavitation process begins, accumulating fluid between granulosa cells. The follicle now is termed as antral follicle or a Graaffian follicle. The Graffian follicles could be healthy follicles differentiating further and finally attaining the preovulatory stage (Erickson et al, 1985). Most follicles do not reach the preovulatory stage, but undergo an apoptotic process of degeneration called

atresia. Most atresia in the adult ovary occurs at the small antral stage of follicular development. Studies have shown that small antral follicles are more susceptible to atresia (Gosden & Laing et al, 1983). The stroma has interstitial glands secreting androgen, sex steroids and growth factors. Antral follicles also produce ovarian steroids.

Based on all the data, it is possible to propose the following cascade mechanism for ovulation. The midcycle surge of LH stimulates the production of progesterone via progesterone receptors (PR); the progesterone ligand interacts with PR in the follicle cells, which induces COX-2 and prostaglandin production; and the prostaglandins interact with specific receptors in the surface epithelial cells of the presumptive stigma. This activates a series of signaling pathways leading to release of the proteolysis enzymes from lysosomes. This process results in stigma formation and follicle rupture (Erickson et al, 1985). After ovulation i.e. the expulsion of oocyte from the follicle, the remaining granulosa and theca layer cells undergo changes called luteinization ultimately forming the corpus luteum. A functional corpus luteum producing progesterone is necessary for the implantation and maintenance of pregnancy. If pregnancy does not occur then the corpus luteum becomes atretic and regresses.

1.8 Toxicity Mechanism

Besides age the speed of follicle depletion can be regulated by genetic, hormonal, and environmental influences. A wealth of experimental data shows that chemotherapy, radiotherapy, environmental toxicants e.g. PAHs and various other ovotoxicants cause premature ovarian failure. Response to the toxicants is

dependent on many factors, including the species, age of subject and the size or type of follicle.

Different follicles have different sensitivities to ovotoxins resulting in different patterns of ovarian failure. A toxin that primarily destroyed preovulatory follicles would result in immediate loss of ovarian function; this can be however restored when surviving less mature follicles continue to develop into new preovulatory follicles. Destruction of growing follicles would result in somewhat delayed loss of function, which, again, might recover, as less mature follicles develop. Conversely if the ovarian reserve or the primordial follicles are destroyed there is permanent loss of fertility as there is no follicle pool left to recruit from. The loss of resting follicles would however be evident only when the pool of preovulatory and growing follicles was exhausted (Mattison et al, 1981).

As discussed in the previous section, one of the mechanisms through which BaP produces toxicity is by forming BaP-DNA adducts, through binding of BaP with cellular macromolecules such as proteins, and nucleic acids (Ramesh & Knuckles et al, 2006). Measurement of these adducts may help us in measuring the biologically effective dose. The relationship between BaP disposition and tissue damage was examined by measuring the concentrations of DNA adducts formed by binding of BaP metabolites with DNA. A progressive decline in the adduct concentrations with increasing periods post BaP exposure is suggestive of an innate adaptive process to cope with toxicant exposure (Ramesh et al, 2010).

Accumulating evidence demonstrates that ROS are key signals in the initiation of apoptosis in antral follicles and granulosa cells of antral follicles by

diverse stimuli, such as gonadotropin withdrawal, exposure to exogenous toxicants, and exposure to ionizing radiation, and that antioxidants protect against these stimuli. GSH in the oocytes is the necessary antioxidant that needs to be present in adequate or higher concentrations during fertilization and embryonic implantation. This suggests that the critical time for embryonic development is very sensitive to exposures, chemical toxicants and environmental stressors that produce oxidative stress (Luderer et al, 2012). Environmental toxicants may also exert their effects by disrupting the communication thru gap junction channels thereby affecting the flow of nutrients to the oocyte from the granulosa cells. This results in subtle changes in the cytoplasmic maturation causing implantation failure or poor embryonic development (Baird et al, 2005). There is no single mechanism that can explain all the toxic effects of BaP on fertility. It is a conjunction of multiple insults and not one single culprit.

2.0 Hypothesis

Transplacental exposure to Benzo [a] pyrene in F1 Muta™mouse results in reproductive consequences.

2.1 Specific Aims

- **To determine if the transgenic Muta™mouse model is a sensitive and appropriate mouse model to study the reproductive consequences of transplacental BaP exposure.**

In the past Muta™mouse, mouse model has generally been used to study genetic

mutations in the somatic and germ cells. Muta™mice have differences in metabolism, DNA repair capacity and proliferation rates among embryonic tissue. Knowing this it is reasonable to assume that the magnitude of BaP toxicity on ovarian follicles of these mice would be different. It will be interesting to see that the *lacZ* trans genetic mutations induced in Muta™mouse make it more susceptible or resistant to BaP toxicity. This is the first time that the Muta™mouse is being used to study the reproductive effects in the F1 generation.

- **To show ovarian follicular depletion in Muta™mouse as a measure of reproductive consequences.**

Number, size and shape of offspring are measures of the reproductive consequences. The ovarian follicle count depletion is another and more accurate measure of reproductive effect (Meirow et al, 1999). We suggest that BaP toxicity in Muta mice will cause follicular depletion, resulting in reproductive consequences.

- **To assess if the ovotoxic effect of BaP is dose dependent.**

As shown in previous various studies, the effects of BaP on the tissues are directly proportional to the dose administered (Lim et al, 2013). Different dose groups will be compared to the control group to see the pattern of follicular depletion.

- **To assess if the reproductive consequences occur in both male and**

female muta mouse.

The F1 generations of male mice after similar exposure and similar experimental conditions have been studied (Marchetti et al, 2015). They concluded that there was a significant decrease in the sperm count, motility and testis weight. This effect was very significant at the doses of 20 and 40 mg/kg BW. *lacZ* analysis was done by Dr. Marchetti & co-workers at health lab Canada. The focus of our experiment is to assess ovarian follicular changes in F1 female Muta™mice from similar exposure.

3.0 Materials and Methods

All chemicals and reagents used for the UC Irvine portions of the experiments were purchased from Fisher Scientific or Sigma Aldrich.

3.1 Animal

The animal selection, treatment, sacrifice, and dissection were performed at Environmental Health Science Research Bureau, Health Canada, Ottawa, ON, Canada under the supervision of Dr. Francesco Marchetti. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Health Science Research Bureau, Health Canada, Ottawa, ON, Canada. Tissues from treated animals were fixed and shipped to UC Irvine from Health Canada. No live animal work was performed at UC Irvine for this study.

For our study Muta Mice were purchased and bred at Health Canada. Eight male Muta Mice were mated with 3-4 female Muta mice. From 7 through 16 post conception days the pregnant mice were fed BaP via oral gavage. The BaP was mixed in olive oil, the doses given were 0, 10, 20, & 40 mg/kg BW/day. Pregnant female mice and F1 offspring were fed rodent chow and provided water ad libitum. The pups were weaned at postnatal day 21. At 10 weeks of age, the F1 females were sacrificed by CO_2 inhalation. Dr. Marchetti and coworkers at Health Canada processed one ovary of female F1 for mutant frequency assays. The other ovary was fixed in Bouin's fixative for 24 h, then washed four times in 50% ethanol for 30-60 min, then stored in 70% ethanol, and shipped to the Luderer laboratory at UC Irvine. The UC Irvine Chao Family Comprehensive Cancer Center Experimental Pathology Tissue Resource embedded the ovaries in paraffin and cut serial sections of 5 μ thickness.

Gossain et al, 1989, first described the Muta™Mouse. When a foreign gene of interest is inserted into another animal, it is termed as transgenic animal. Muta Mouse was constructed originally by microinjection of the bacteriophage λgt10-*lacZ* shuttle vector into fertilized CD2 mouse oocytes. The vector is approximately 47 kB in length and contains *lacZ* (3100 bp) and cII as reporter genes. *lacZ* is one of the genes on plasmids, which are small portions of DNA. *lacZ* encodes for beta-galactosidases used by E.coli to digest lactose. Plasmids are used as vectors to insert genes into host cells. cII reporter gene is a similar but much easier and faster way to see effects. It uses the positive selection method in which only the mutant colonies grow on the selection plates, whereas in *lacZ* assay the mutant colonies are

identified by color changes.

Muta mouse carries 40 copies of the shuttle vector in the head to tail array at a single site in chromosome 3 (Gossen et al, 1989). Homozygous mice with 80 copies of the shuttle vector are commercially available. After exposure of the Muta Mice to the test substance, (in our study BaP), DNA can be isolated from individual organs or tissues, and a single copy of the vector DNA can be excised from the high molecular weight rodent DNA and transferred into infectious particles. When appropriate E coli. host cells are infected, plated, and incubated, plaques or colonies containing individual transgene vectors become visible on plates.

The genomic DNA was isolated from multiple tissues, including ovary, bone marrow, sperm, and liver as per the OECD protocol. *LacZ* transgene mutants present in the genomic DNA were identified using the Phenyl-β-D-galactopyranoside (P-gal) positive selection assay. Briefly, the λgt10lacZ construct was packaged into phage particles through the cohesive ends (cos sites) using the Trans pack TM lambda packaging system (Agilent, Santa Clara, CA). Packaged phage particles were subsequently mixed with the host bacterium (E. coli *lacZ*) in order to infect the E. coli with the *lacZ* construct. The E. coli were then plated on minimal medium containing 0.3 % (w/v) P-gal and incubated overnight at 37 °C. P-gal is toxic to galE strains that express a functional copy of *lacZ*, and thus, only E. coli receiving a mutated copy of the *lacZ* transcript will form plaques on the P-Gal medium. Packaged phage particles were concurrently plated on titer plates without P-Gal to determine the total plaque-forming units (pfu). The *lacZ* mutant frequencies were then calculated by determining the ratio of mutant pfu to total pfu (Marchetti et al,

2015).

Muta^{TM}Mouse Transgenic Rodent Assay

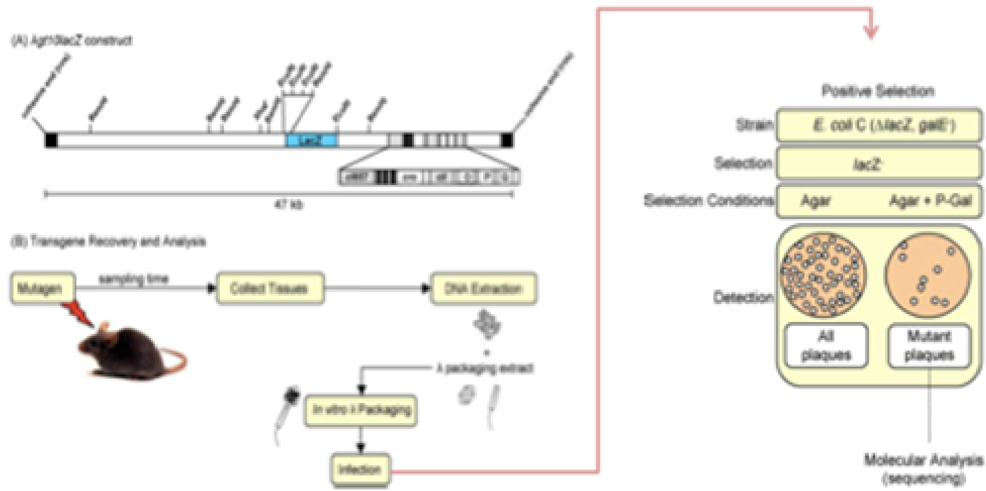


Figure: 2 -Trans placental Mutagenicity of Benzo (a) pyrene in the mouse

(Marchetti et al, 2015)

3.2 Histological staining

Two investigators Dr. Luderer and Dr. Meharda performed Histochemical staining with hematoxylin and eosin and enumerated ovarian follicles for 23 ovaries blind to treatment group. Staining was done as per a standard protocol. The slides were first deparaffinized by washing in Histoclear twice on a rocker platform for 10 minutes. This was followed by washing with 100% ethanol, 95 % ethanol, and tap water three times each in order to rehydrate the paraffin embedded slides. The slides were then incubated in the Gill's 3 hematoxylin without rocking for 10 minutes, washed with water, then 0.4% HCL-95% alcohol, and water again, and 0.1% Ammonium hydroxide treatment to remove the excess, and to enhance the blue nuclei in the slides. Slides were further incubated in 1% eosin Y stagnant for 4 minutes. The slides were finally dehydrated through 3 washes with 100 % ethanol, 3 washes with 95% ethanol, and 3 washes with Histoclear. They were cover slipped with Omni mount & dried overnight under the fume hood, followed by additional drying for at least several days.

3.3 Follicle counting

The follicles were counted using an Olympus BX60 microscope equipped with Plan Fluor ×10, ×20, and ×40 objectives, Plan Achromatic ×4 objective (Olympus America).

Ovarian follicles were classified as primordial if single layer of flattened granulosa cells were present around the oocyte. If single layer of cuboidal cells were

present, or mixed cuboidal with flattened granulosa cells were present they were classified as primary follicles. When more than one layer of granulosa cells were present around the oocyte they were classified as secondary follicles. Follicles with an antral cavity or several fluid filled vesicles within the granulosa cell layers were classified as antral follicles.

Follicles were further classified as healthy or atretic. The presence of 3 or more pyknotic granulosa cells per largest cross section of secondary and antral follicles classified them as atretic follicles. Atretic primordial and primary follicles were identified by eosinophilic oocytes (Hirshfield et al,1988).

Primordial, primary, and secondary follicles were counted in every fifth serial section. The counts were multiplied 5 times to obtain estimates of the total number of follicles per ovary. Antral follicles were followed through every serial section, taking care to count each of these structures only once (Luderer & Jin et al, 2012).

4.0 Statistical Analysis

The end point of the study was various differential ovarian follicle counts (primordial, primary, secondary, antral, cysts, corpora lutea). The follicle counts were the continuous outcome variables and BaP doses were the fixed independent variable. BaP dose group used one-way ANOVA to assess differences in the outcomes. Post Hoc tests (BTUKEY DUNCAN LSD T3 DUNNETT) were used for pairwise comparisons of treated groups with the control group. The means and SEMs were used for presentation of the data. Statistical analyses were conducted using IBM SPSS version 23 for Windows.

5.0 Results

In total 23 ovaries were obtained from 23 F1 female Muta Mice. There were 11 ovaries from the control group, 6 ovaries from the group treated with 10 mg/kg BW BaP, 4 ovaries from the group treated with 20 mg/kg BW BaP, and 2 ovaries from the group treated with 40 mg/kg BW BaP.

Table A shows the counts of healthy and atretic primordial, primary, secondary, and antral follicles, as well as corpora lutea and cysts per ovary for each individual mouse. As expected prenatal BaP decreased follicle numbers at all stages of follicular development, starting at the lowest dose of 10 mg/kg BW. The individual variation in the primordial, primary and secondary follicle counts in the control group was also striking, but expected based on prior studies from the Luderer laboratory and other groups.

Table A: Individual ovarian follicle counts in ovaries of 23 F1 female Muta Mice, exposed to 0, 10, 20, or 40 mg/kg/day BaP to their mothers on day's 7- 16-post conception

Ovary ID	Treatment (mg/kg)	Primordial	Primary	Secondary	Antral	Corpus Luteum	Cyst	Primordial Atretic	Primary Atretic	Sec Atretic	Antral Atretic
OV26Cf1	0	1725	855	114	67	4	0	0	0	1	4
OV11lf	0	2860	775	83	43	2	2	0	0	0	19
OV9lf	0	1640	500	48	39	17	0	0	0	6	8
OV29 CFI	0	1680	720	37	0	19	0	0	0	0	0
0V3if	0	1075	510	82	78	33	55	0	0	0	12
OV7if	0	1040	385	64	71	29	17	0	0	16	9
0V5if	0	485	205	21	55	31	42	0	0	6	15
OV30cf1	0	810	355	49	141	62	24	0	0	0	34
OV1if	0	950	315	38	121	64	24	0	0	0	21
0V23Cfi	0	870	350	39	111	60	4	0	0	0	16
0V27Cfi	0	425	120	11	65	36	18	0	0	0	9
OV 41 f	10	0	0	0	0	0	0	0	0	0	0
OV10if	10	430	175	7	23	12	41	0	0	0	12
OV2if	10	480	195	16	27	16	42	0	0	0	12
OV6if	10	215	60	0	3	20	0	0	0	0	8
OV8if	10	165	70	0	7	21	22	0	0	0	12
OV12if	10	235	80	10	55	33	11	0	0	0	19
OV26Bfi	20	0	0	0	0	0	0	0	0	0	0
OV28Bfi	20	70	30	0	0	0	0	0	0	0	5
OV34Bfi	20	10	10	0	1	0	0	0	0	0	2
OV30bf1	20	255	100	0	0	0	0	0	0	0	0
OV30Afi	40	30	0	0	0	0	0	0	0	0	0
OV27Afi	40	95	0	0	0	0	0	0	0	0	0

The primordial follicles were statistically significantly reduced by 80% at the treatment dose of 10 mg/kg BW (P<0.005 compared to 0 mg/kg). Similarly at the dose of 20 mg/kg BW the primordial follicles were reduced by 94% (P<0.001), and at 40 mg/kg BW they were reduced by 98% (P<0.001). There was a very obvious

dose dependent decrease in the primordial follicle count (Fig. 3). These finding show that the ED50 for primordial follicle destruction by prenatal BaP is less than 10 mg/kg BW/day during the developmental stage from gestational days 7-16 (Fig. 3).

The number of primary follicles was decreased by 80 % at the dose of 10 mg/kg BW/day, by 92% at 20 mg/kg BW, and by almost 100 % at the dose of 40 mg/kg BW when compared to the control group (Fig. 4). The number of corpora lutea declined significantly (50%) at the 10 mg/kg BW dose; there were no corpora lutea in the 20 or 40 mg/kg dose groups.

Cysts were the only ovarian structures that were increased in the 10 mg/kg BW as compared to the control group (Fig. 8). The ovarian follicles when destroyed are replaced by disorganized fibrosis and cyst formation. The cysts therefore signify follicular or ovarian destruction.

Table B: Differential mean follicular count in control and treatment group with standard error and standard deviation.

(M=Mean; SD=Standard Deviation; SE=Standard Error)

BaP Dose [mg/kg BW]	**0**			**10**			**20**			**40**		
	M	**SD**	**SE**	**M**	**SD**	**SE**	**M**	**SD**	**SE**	**M**	**SD**	**SE**
Primordial	1233	701	211	254	177	72	84	118	59	63	46	33
Primary	463	236	71	97	74	30	35	45	23	0	0	0
Secondary	53	30	9	6	7	3	0	0	0	0	0	0
Antral	72	40	12	19	21	8	0	1	0	0	0	0
Corpus Luteum	32	22	7	17	11	4	0	0	0	0	0	0
Cyst	17	19	6	19	19	8	0	0	0	0	0	0

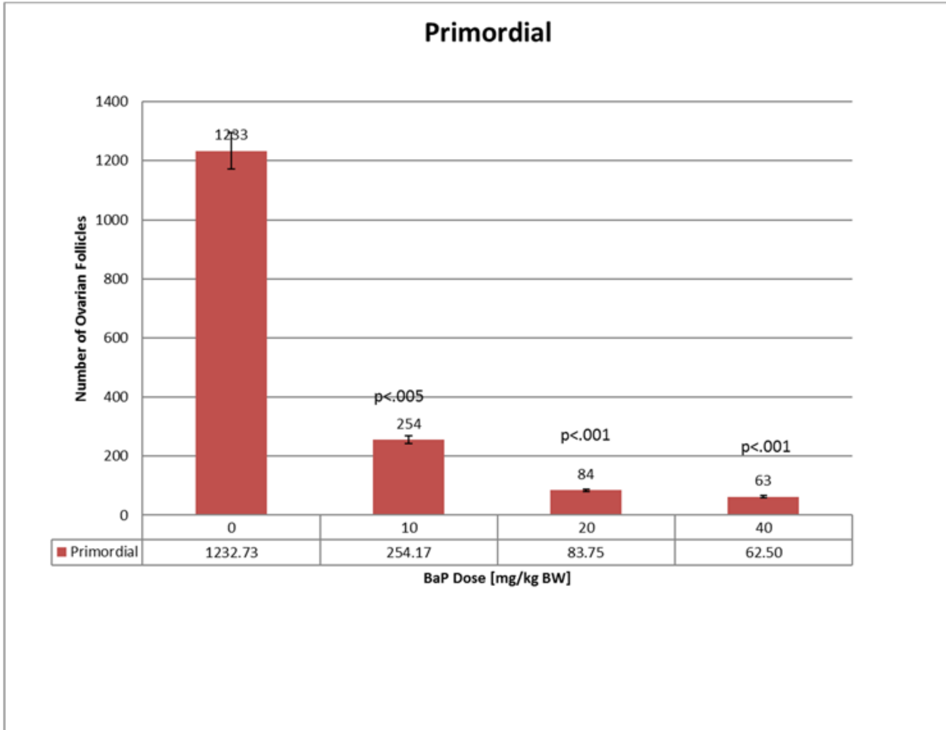


Figure: 3 - Total Number of Primordial Ovarian follicles in F1 female Muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days.

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

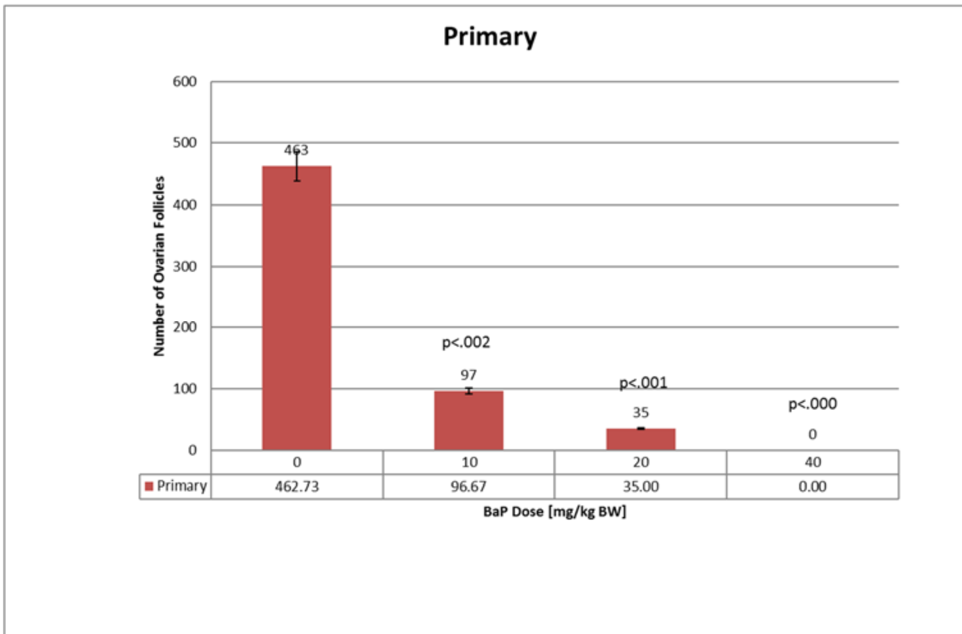


Figure: 4 - Total Number of Primary Ovarian follicles in F1 female Muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

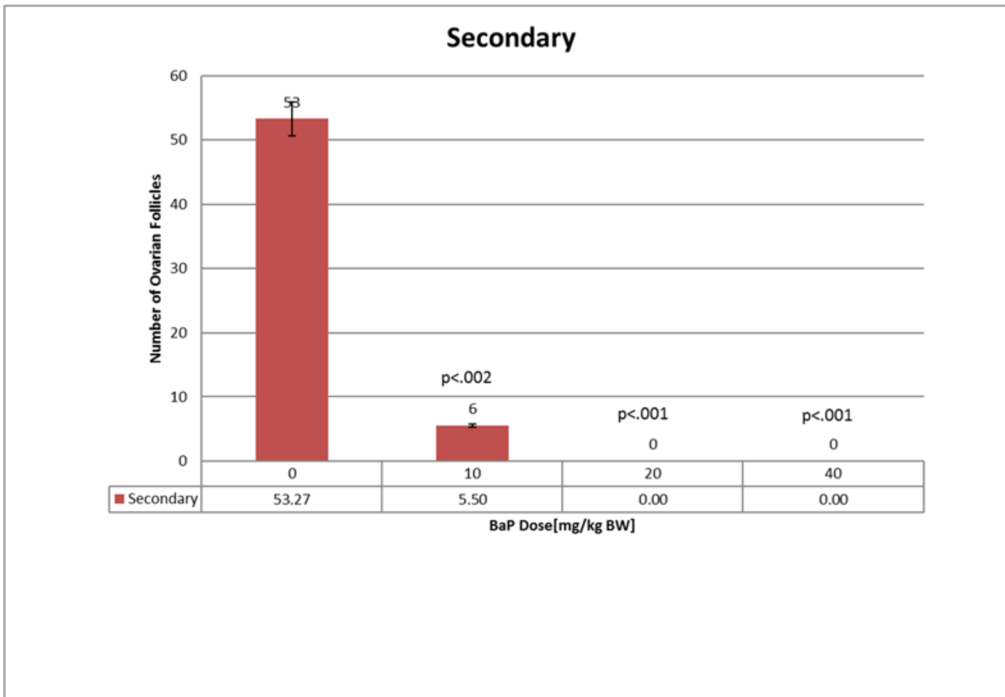


Figure: 5 - Total Number of Secondary Ovarian follicles in F1 female muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

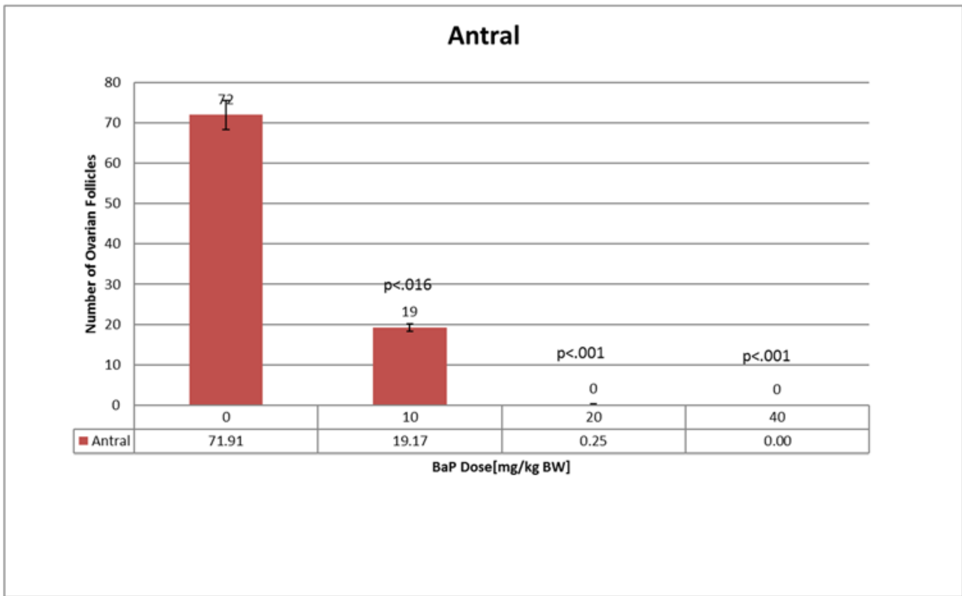


Figure: 6 - Total Number of Antral Ovarian follicles in F1 female muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

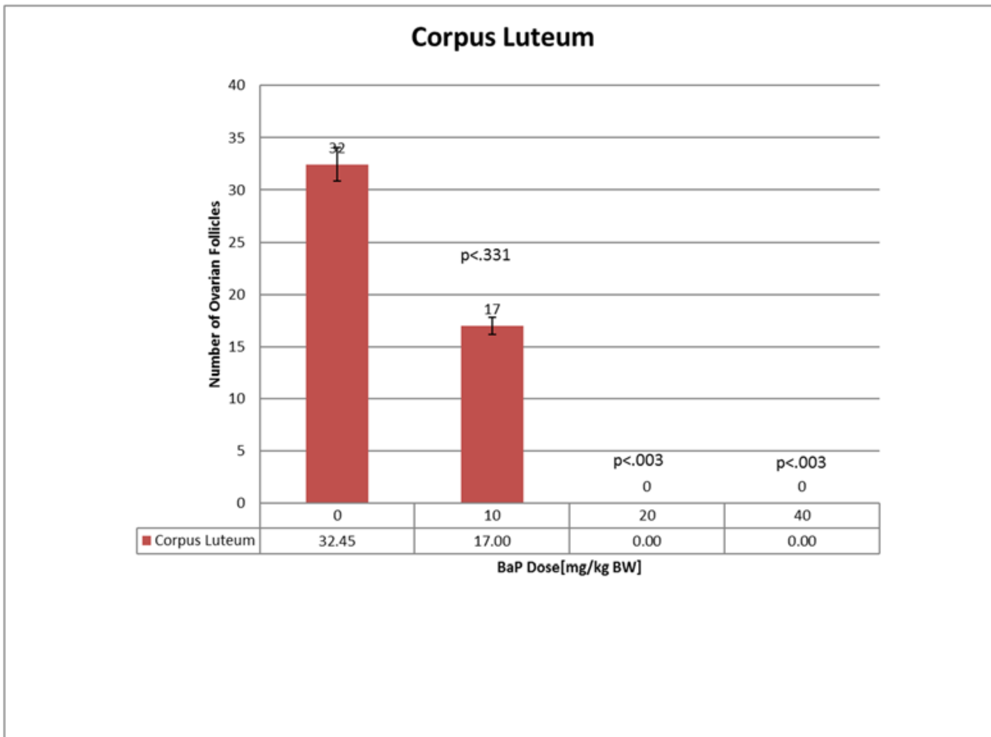


Figure: 7 - Total Number of Corpora Lutea in F1 female muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

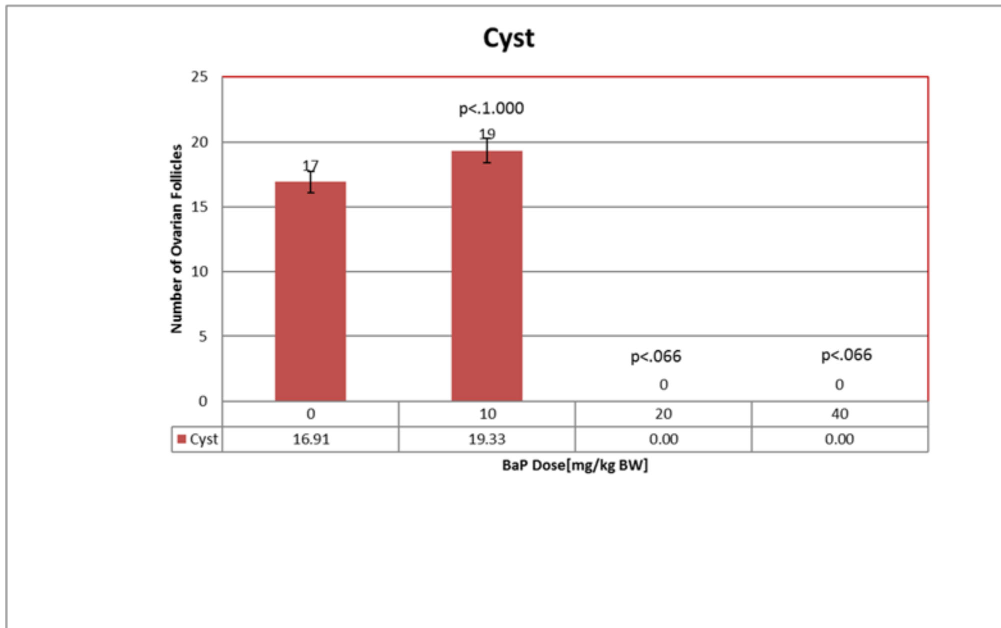


Figure: 8 - Total Number of Cyst in F1 female muta mice when exposed to different treatment doses of BaP from 7th through 16th post conception days

P<0.001, overall effect of treatment by ANOVA. The p-values shown are for pairwise comparisons with the 0 mg/kg group using Dunnett's T3 test.

To summarize, our results show that prenatal exposure to BaP resulted in dose-dependently lower numbers of all different kinds of follicles at 10 weeks of age when compared to the control group. Even though the ovarian weight or size was not changed from the control group (Marchetti et al, 2015), the follicular numbers were drastically different from the control group. The 20 and 40 mg/kg BW/day doses of BaP destroyed almost all the follicles. The ovarian anatomy at higher doses was difficult to recognize, as most of the healthy tissue was replaced by fibrosis.

6.0 Discussion

Loss of primordial and primary follicles has profound biological consequences. It is generally accepted that mammals are born with a finite number of primordial follicles, the loss of oocytes over time with normal ovarian aging appears to be irreversible, ultimately resulting in depletion of resting ovarian pool and sterility (Jurisicova et al, 2007).

BaP is a known environmental toxicant, which has been studied extensively as a causative agent for follicle disruption. Both primary and secondary are targeted.

To our knowledge this is the first time that Muta Mice have been used as experimental animals to study the follicular depletion leading to reproductive consequences in F1 generation after BaP exposure. This strain of mice is generally used for gene mutation and tumorigenesis studies.

- **Reproductive consequences seen in the male counterparts F1 mice with similar exposure**

  Our study is a sister study of the experiment done by Dr. Marchetti and co-workers at Environmental Health Science Research Bureau, Health, Canada. The male siblings of the females in the present study have been studied for the reproductive consequences and mutagenesis in testis after the similar BaP exposure. Their results showed that, in-utero exposure to 0, 10, 20, and 40 mg/kg BW/day BaP resulted in no significant changes in litter size or body weight of F1 animals, this includes the female mice used for our study. However

high doses of BaP (20 and 40 mg/kg BW/day) significantly decreased sperm count, sperm motility, and testis weight in F1 males exposed prenatally.

Mutant frequencies in sperm of F1 males exposed in-utero to BaP (20 mg/kg BW/day) were increased ($p<0.0001$) with respect to controls. Although this increase was significantly lower ($p<0.01$) than the frequencies observed in somatic tissues, but mutations in sperm are potentially heritable, and therefore may affect future generations as well. Mutant frequencies were also significantly increased, in a dose-dependent manner, in the bone marrow, liver, and brain of F1 males exposed in-utero to BaP.

Their results also showed that in-utero exposure to BaP induces mutations in somatic tissues as well as germ cells. The magnitudes of induced mutant frequencies are lower in germ cells; however, primordial germ cell precursors seem to be more susceptible to apoptosis (Marchetti et al, 2015).

- **Muta™mice strain is very sensitive to the ovotoxic effects of BaP**

Previous studies have shown that different strains of mice have different thresholds to the toxic effects of BaP and its metabolites. Bolon, (1997), hypothesized that follicle count is a more quantifiable, objective and sensitive estimate of ovarian toxicity as compared to ovarian weight or fertility function. Even though the objective of their study was to study chemical toxic (not BaP) effects on 3 different strains of mice (CD1, C3H, C57BL/6), some important conclusions were derived from their study. They found that there was a large

variation in differential follicle counts between control mice from different strains. Both C3H and C57BL/6 mice are less fecund than CD-I mice (Chapin et al, 1993). In their analysis, the two inbred strains had three to four fold fewer follicles of each class than did age matched CD-I mice.

In another experiment by Lim et al, (2013), 10 week old C57BL/6J mice strain were used. F0 females were given oral dose of 0, 2, and 10 mg/kg/d, BaP daily from GD7–16. Each Gclm-/-(glutamate cysteine ligase modifier subunit], and Gclm +/+ F1 female offspring was continuously bred with a wild-type male for 20 weeks beginning at 8 weeks of age. In utero treatment with 10 mg/kg/d BaP profoundly decreased fertility of female mice of both genotypes. Whereas treatment with 2 mg/kg/d BaP decreased fertility to a much greater extent in Gclm-/- females than in Gclm +/+ females. Two of two 10 mg/kg/d BaP-treated Gclm-/- mice and 4 of 6 Gclm +/+ 10 mg/kg/d BaP-treated mice delivered no litters and showed no signs of pregnancy during the study period. Exposure to BaP from GD 7 to 16 doses dependently decreased total number of healthy ovarian follicles from 2,250 ± 348 in 0 mg/kg BaP-exposed, to 1,036 ± 338 in 2 mg/kg BaP- exposed, and 34 ± 16 in 10 mg/kg BaP-exposed (Luderer & Lim et al, 2012).

Consistent with the above-mentioned studies our experiment also showed more than five fold loss of the small follicles (primordial and primary) at the lowest dose of 10 mg/kg BW/day after prenatal BaP exposure. It could be possible that the Muta Mice strain, similar to Gclm deficient mice is very

sensitive to the ovotoxic effects of BaP. *LacZ* analyses of ovarian DNA performed by our collaborator Dr. Marchetti showed that mutant frequencies were significantly increased, in a dose-dependent manner, in the other ovaries of the same F1 females exposed in-utero to BaP that we used in the present study (unpublished observations).

- **Ovarian Follicular counting is a more accurate reflection of toxicity.**

Meirow et al, (1999) conducted an experiment on 5-6 week old Balb/c mice. 29 mice were treated with 20, 50, 75 and 100 mg/kg BW of cyclophosphamide (chemotherapeutic agent) injection for 7 days. The follicle counting showed a dose dependent decrease in the primordial follicle counts. At 75 mg/kg BW almost 54% of primordial follicles were lost. In the second phase of the experiment 101 young female mice (5-6 week old) were treated with 75-mg/kg BW cyclophosphamide. It was seen that the ovulation, mating and pregnancy outcome of the treated mice was not compromised as compared to the controls. Thus indicating that reproductive performance is not an accurate parameter for assessing ovarian injury. Rather, histological counting of ovarian follicle number more directly reflects the damage caused by toxic agent to the ovary (Meirow et al, 1999).

In our experiment 80% reduction of the primordial and primary follicles was seen after the treatment dose of 10 mg/kg BW/day prenatally. This clearly is consistent with the prior studies that prenatal exposure to BaP causes toxic

effects in the ovary, which can be measured in the terms of reduction of follicle counts.

- **BaP toxicity has dose dependent response**

Our results show that all the follicles in different groups depleted in a dose dependent manner. A linear decline was seen starting at the minimum experimental dose of 10 mg/kg BW, which continued to almost total destruction of ovarian tissue at the maximum dose of 40 mg/kg BW. Our results are consistent with the study done by Lim et al, (2013). In their experiment the same dose dependent linear relationship was seen. The treatment doses used in their experiment were 0, 2 and 10 mg/kg/d from gestational day 7 to 16. Treatment with 10 mg/kg/d BaP profoundly decreased fertility of female mice of both genotypes. The Gclm deficient mice were more sensitive therefore decreasing the fertility to a much greater extent. At 7.5 months of age, neither *Gclm* null or wild type mice treated with 10 mg/kg/day in utero had any ovarian follicles remaining in the ovaries, while *Gclm* null females exposed to 2mg/kg/day had significantly greater follicle depletion than the wild type mice (Lim et al, 2013). Overall, the ED50 for follicle depletion in their study was about 2 mg/kg/day. Though the strain of mice in our study was Muta mouse, the pattern of follicular depletion was similar between the two studies.

Another study done on peripubertal and adult mice by Mattison et al, (1979,1980) also showed consistent results with our study. In their experiment they showed that BaP, 9,10 dimethyl-1, 2-benanthracene (DMBA) and 3-methylcholanthrene (3-MC) are potent ovarian toxicants, which dose

dependently destroy primordial and primary follicles in peripubertal mice and rats after single high dose (Mattison et al, 1979,1980) or repeated low doses (Borman et al, 2000).

- **Reproductive consequences are seen in both male and female mice**

Male Muta Mice (sister study) showed decreased sperm counts, decreased sperm motility and increased mutant frequencies in the epithelial germ cells (Marchetti et al, 2015). Female Muta Mice showed very significant decreases in all developmental stages of ovarian follicles. Ovarian tissue was destroyed and replaced by fibrosis at higher doses. The ovarian follicular depletion in the Muta mouse strain is consistent with the results of Lim et al, (2013). In their study, in utero treatment with 10 mg/kg/d BaP profoundly decreased fertility of female mice of both Gclm+/-mice genotypes. Whereas treatment with 2 mg/kg/d BaP decreased fertility to a much greater extent in Gclm-/- females than in Gclm +/+ females.

Our observation is also consistent with the study done by Mackenzie and Angevine, (1981). In their study male and female mice were exposed in utero to 40 or 160 mg/Kg maternal BW per day from 7-16th gestation day. This resulted in marked alteration in gonadal morphology and germ cell development. There was 60 % reduction in the reproductive capacity of female mice when exposed to 10 mg/kgBW. In male mice there were atrophic seminiferous tubules and pronounced increases in interstitial cells. The testicular damage at 10 mg/kg BW was partial. Our results are similar to the above-mentioned studies showing that

BaP exposure in-utero results in both ovarian follicular depletion and male gonadal functional changes.

- **Toxicity in F1 generation is a result of placental permeability to BaP and metabolites**

The placenta is very permeable to BaP. Our study is in confirmation with the experiment done by Lim et al, (2013) where F0 mice were exposed to BaP during the same developmental period and by the same route as in our study i.e. 7-16 GD.

A single dose of 200 mg/kg BW (Alexandrov et al, 1976) was administered in pregnant mouse dams at 21st day of gestation. Placentas were highly permeable and showed maximal accumulation of BaP after 3 hours. After 5 hours only minimal BaP was left. It suggested that the effects of BaP on the F1 generation are due to the placental transmission to the fetus rather than transmission through lactation.

- **ED50 of prenatal BaP exposure from gestational day 7 to 16 in Muta mice is less than 10 mg/kg BW/day**

More than 50 % of follicles were depleted at the dose of 10 mg/kg BW in our experiment. This leads to a conclusion that the ED 50 for follicle destruction of BaP is less than 10 mg/kg BW/day. Our results are supported by the evidence seen in the study done by Lim et al, (2013). The mouse strain used in their study was different as compared to our study (C57BL/6J versus Muta mouse) but the

follicle counts and fertility of both genotypes of mice in their study was greatly reduced at the dose of 10 mg/Kg/d. In the glutathione deficient mice the follicle counts and fertility was even further reduced, starting at the dose of 2mg/Kg/d.

Our results in the Muta mouse strain regarding ovarian follicle depletion by prenatal exposure to BaP, with an ED50 less than 10 mg/kg/day, are similar to the Lim et al, (2013) study, which found an ED50 of about 2 mg/kg/day in the C57BL/6J mouse strain.

Our data showed 50 % reduction in the corpora lutea and almost all of the ovarian tissue replaced by connective tissue at doses 20 and 40 mg/kg BW. This is consistent with the findings of Mackenzie and Angevine, (1981). Their study showed that at 40 and 120 mg/kg BW/day the F1 female mice either had no ovaries or only remnants of ovarian tissue. The examination of ovarian tissue revealed that the ovaries were hypo plastic and had very few follicles or corpora lutea at the 10 mg/kg/day dose. At the dose of 40 mg/kg there were absolutely no follicles just as seen in our study. Even though the ovarian weight did not change, the follicles were replaced by the connective tissue further destroying the reproductive capacity of the ovary (Boron et al,1997).

7.0 Conclusion

Ovotoxicants, such as BaP elicit an ovarian response that includes activation of xenobiotic metabolism, resulting in dramatic ovarian germ cell depletion in the developing embryo/fetus or ovarian follicular destruction postnatally. BaP is freely permeable through the placenta thereby causing deleterious effects not only in the F0 generation but also in the F1 generation. The ovarian follicular pool is very sensitive to the detrimental effects of BaP. Any insult to the ovarian reserve results in temporary or permanent sterility. Our study confirms the ovotoxic effects of prenatal exposure to BaP. It also adds that Muta Mice are very sensitive to ovarian germ cell depletion by prenatal exposure to BaP. They can be used as excellent mouse models for not only mutation studies but also for reproductive toxicological studies.

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