# *VI- Conclusiones*

Nuestros resultados demuestran que la exposición prenatal a dosis bajas de BPA modifica la histoarquitectura de la glándula mamaria y aumenta la susceptibilidad a un carcinógeno químico, administrado 50 días después de que ha finalizado la exposición al PE.

Aunque es prematuro concluir que el BPA es carcinogénico por sí mismo, nuestros resultados brindan evidencias para señalar que:

a) La exposición perinatal a BPA aumenta la incidencia de lesiones pre-neoplásicas de la glándula mamaria en la vida adulta.

b) La exposición prenatal a dosis ambientalmente relevantes de BPA altera la histoarquitectura de la glándula mamaria, mostrando signos considerados marcadores de riesgo de cáncer de mama en humanos.

c) La mayor angiogénesis, la fibrosis en el estroma y el incremento en el número de mastocitos asociados espacialmente con los conductos hiperperplásicos, pueden tener un rol permisivo, o aún co-causal, en la carcinogénesis inducida por NMU.

d) La asociación entre la exposición prenatal a BPA y posnatal a una dosis sub-carcinogénica de un carcinógeno químico promueve la progresión de lesiones pre-neoplásicas a neoplasias.

Los resultados presentados en esta tesis son consistentes con nuestra hipótesis de que "la exposición *in utero* a BPA genera cambios histomorfológicos en la glándula mamaria que podrían modificar la regulación endocrina y aumentar la susceptibilidad a un carcinógeno químico".

Consideramos relevante preguntarnos cuál es la implicancia de estos resultados obtenidos usando un modelo ampliamente aceptado para imitar la carcinogénesis mamaria en humanos. Nuestras observaciones refuerzan los argumentos que asocian el aumento en la incidencia de tumores hormonodependientes en humanos con la exposición *in utero* a dosis mínimas de PEs (como BPA), a los cuales las mujeres embarazadas están cotidianamente expuestas.

Creemos que es necesario replantear la discusión de las dosis consideradas "seguras" como medida preventiva y adherimos al principio de precaución, redactado en la Conferencia de Wingspread en 1998:

"Cuando una actividad emergente represente un daño para la salud humana o del medio ambiente, deben tomarse medidas de precaución aunque la relación causa-efecto no esté completamente demostrada con rigor científico".

# *VII- Resumen*

Los perturbadores endocrinos (PEs) están definidos como "una sustancia o mezcla de sustancias exógenas que altera una o más funciones del sistema endocrino y en consecuencia causa efectos adversos en la salud de un organismo intacto, su progenie o subpoblaciones". Algunos PE imitan las acciones del 17β-estradiol ($E_2$) y son denominadas genéricamente **xenoestrógenos.** Se hipotetizó que en humanos la exposición a xenoestrógenos, podría aumentar la incidencia de tumores hormonodependientes. Uno de los **xenoestrógenos** más estudiados es el bisfenol A (BPA), usado en la manufactura de policarbonatos plásticos, resinas epoxi y selladores odontológicos. El riesgo de exposición a BPA es potencialmente elevado debido al uso cotidiano de productos que lo contienen (envases de plástico de bebidas y alimentos, mamaderas, discos compactos, lacas y pinturas). El BPA se libera por exposición al calor o frente a ácidos o álcalis fuertes. En nuestro laboratorio estudiamos los efectos de la exposición perinatal a BPA, no sólo en roedores de laboratorio, sino también en especies de interés zootécnico y de la fauna nativa.

La glándula mamaria (GM) es el órgano que define a la Clase Mamíferos y su función principal es sintetizar y secretar la leche que servirá para nutrir a las crías recién nacidas. El desarrollo de la GM comienza en la vida uterina y se completa durante la vida adulta. Diversas hormonas y factores de crecimiento controlan el desarrollo mamario. La GM un órgano hormonodependiente. La rata posee seis pares de glándulas mamarias, dispuestas bilateralmente en la zona ventral, desde la región cervical hasta la región inguinal. El parénquima mamario está formado por una red de conductos que transportan la leche desde su lugar de síntesis (alvéolos) hacia el pezón. Los conductos principales o mayores poseen ramificaciones dando origen a conductos más pequeños denominados conductillos. Las estructuras más primitivas o inmaduras son los denominados *terminal end buds* (*TEBs*); *terminal ducts* (*TDs*) o *blunt end buds* y *alveolar buds* (*ABs*). Cada estructura epitelial está inmersa en una capa de tejido conectivo y todo está rodeado por tejido adiposo, denominado *fat pad* que se encuentra cubierto por una cápsula de tejido conectivo. El desarrollo del árbol ductal depende de las interacciones estroma-epitelio, importantes tanto durante el desarrollo embrionario como posnatal.

Los esteroides ováricos (Eg y Pg) poseen una reconocida capacidad para actuar como reguladores del crecimiento y la diferenciación celular en diversos órganos y tejidos, ejerciendo su acción a través de sus receptores: receptor para estrógenos (RE) y para progesterona (RP), junto con otros factores de transcripción y co-reguladores (co-activadores y co-represores). Los Eg son mitógenos potentes en la GM, necesarios tanto

para el desarrollo normal como para la inducción y progresión de carcinomas mamarios. En la GM, la vía de señalización Pg-RP, está implicada en la ramificación del árbol ductal (*branching*) y el desarrollo lobuloalveolar.

Estudios epidemiológicos muestran un aumento en la incidencia de tumores hormonodependientes en general y de mama en particular que coincide con la introducción de químicos clasificados como PE en el ambiente.

Existen tres teorías en relación al inicio del cáncer: 1) la **teoría de las mutaciones somáticas;** 2) la **teoría epigenética;** 3) la **teoría de la organización tisular.** Los cambios genéticos asociados con la carcinogénesis alteran propiedades fundamentales de las células permitiendo que éstas evadan los controles normales del crecimiento y finalmente adquieran el fenotipo de una célula cancerosa. Las teorías epigenética y de la organización tisular explicarían mejor la relación entre exposición a PEs y cáncer.

En el proceso de carcinogénesis hay dos componentes principales: el carcinógeno y el tejido blanco. Un gran número de agentes produce daño genético e induce la transformación neoplásica de las células. El cáncer de mama es el tumor más diagnosticado entre las mujeres y es la segunda causa de mortalidad por cáncer. La exposición a Eg a lo largo de la vida es un factor de riesgo para el desarrollo de cáncer de mama. Estudios epidemiológicos sugieren una fuerte correlación entre exposición a Eg y/o xenoestrógenos y cáncer y numerosos estudios experimentales tanto *in vivo* como *in vitro* apoyan esta asociación. En los últimos 50 años, se utilizaron diferentes modelos para estudiar la biología del cáncer y para desarrollar y evaluar estrategias terapéuticas y de prevención. Los dos modelos de carcinogénesis química más utilizados son los que emplean DMBA (7,12-dimethylbenz($\alpha$)anthracene) y NMU (*N*-methyl-*N*-nitrosourea). Existe una correlación entre los cambios inducidos por el carcinógeno y la estructura de la GM al momento de la administración de la droga. La susceptibilidad de la GM es máxima cuando el carcinógeno es administrado a 45-60 días de edad, que coincide con el período en el cual los *TEB*s están activamente diferenciándose a *AB*s. Una vez administrado el carcinógeno, los *TEB*s presentes en la glándula se transforman en proliferaciones intraductales, hiperplasias ductales o conductos hiperplásicos, que reciben la denominación de lesiones pre-neoplásicas por considerarse a esta transformación el primer paso en el proceso de tumorigénesis mamaria. Las lesiones pre-neoplásicas pueden progresar a carcinoma ductal *in situ* y luego a adenocarcinoma. Las características del estroma circundante, infiltración de mastocitos y neovascularización estarían estrechamente vinculadas a la progresión tumoral.

Nuestra hipótesis propone que la exposición a bajas dosis de un xenoestrógeno durante períodos críticos de la histiogénesis/organogénesis/diferenciación de la GM produce cambios permanentes a nivel del genoma los que se manifiestan con alteraciones en el equilibrio proliferación/apoptosis y/o el microambiente tisular favoreciendo el proceso de la carcinogénesis.

**Objetivos específicos**

En ratas Wistar, estudiar si la exposición *in utero* a bajas dosis de BPA, altera una serie de parámetros en las crías hembras:

- modifica parámetros de crecimiento corporal e inicio de la pubertad;
- altera el desarrollo histomorfológico de la GM;
- perturba la histoarquitectura del estroma mamario;
- modifica la infiltración y/o presencia de células inmunocompetentes en la GM;
- modifica la esteroidogénesis ovárica generando cambios en los niveles séricos de esteroides ováricos;
- modifica la expresión de receptores para hormonas esteroideas y de co-reguladores en la GM;
- modifica parámetros relacionados con la angiogénesis mamaria;

En ratas hembras expuestas *in utero* a BPA y posnatalmente a NMU se estudiará:

- la sensibilidad de la GM a la acción de un carcinógeno químico;
- la histomorfología del parénquima y estroma mamarios;
- la incidencia y multiplicidad de tumores de mama y de lesiones preneoplásicas;
- el tipo de los tumores generados, de acuerdo con su histomorfología y con la expresión de receptores hormonales.

**Materiales y Métodos**

Para alcanzar los objetivos propuestos se diseñaron experimentos en dos etapas. En la **ETAPA I** evaluamos los efectos de la exposición prenatal a BPA sobre: 1) parámetros de crecimiento y desarrollo de las crías; 2) desarrollo de la GM; 3) niveles séricos de hormonas esteroideas; receptores hormonales y sus co-reguladores en la GM; 4) parámetros de angiogénesis; en diferentes etapas de la vida posnatal. En la **ETAPA II**, evaluamos: 1) la susceptibilidad de la GM frente a un carcinógeno químico, 2) si la exposición prenatal a BPA modifica la respuesta de la GM al carcinógeno.

Ratas Wistar recibieron a través de bombas osmóticas sc, desde el día de gestación 8 (DG8) hasta el parto: vehículo (dimetilsulfóxido, DMSO), o BPA en tres dosis diferentes: 25 μg/kg/d; 250 μg/kg/d; 25 mg/kg/d (25 BPA, 250 BPA y 25 mBPA, respectivamente). **ETAPA I**: la distancia ano-genital fue evaluada en hembras neonatos; todas las crías hembras fueron pesadas durante la lactancia y en distintos momentos de la vida adulta; el inicio de la pubertad fue evaluado según el criterio de apertura vaginal. Las crías hembras se sacrificaron a los 30, 50, 110 ó 180 días de edad, considerados días posnatal (DPN 30, 50, 110, 180). **ETAPA II:** mediante dos pruebas pilotos (para evitar el uso excesivo de animales) se evaluó la susceptibilidad al cacinógeno NMU en hembras inyectadas en DPN 21 (pre-púberes) *vs* DPN 50 (post-puberales) y se seleccionó una dosis sub-carcinogénica de NMU. Una vez optimizado el modelo, un grupo de crías expuestas *in utero* a DMSO o BPA fue inyectado en DPN 50 con 25 NMU (dosis sub-carcinogénica) y sacrificado en DPN 110 o DPN 180. Como control de actividad del carcinógeno, un tercer grupo de hembras expuestas *in utero* a DMSO, recibió en DPN 50 una dosis carcinogénica de NMU (50 mg/kg, 50 NMU) y fue sacrificado en DPN 180. Dos horas antes del sacrificio todos los animales recibieron bromodeoxyuridina. Se obtuvieron muestras de suero y ambas cadenas mamarias abdomino-inguinales. Una cadena mamaria fue procesada como *whole-mount* para la detección de lesiones micro y/o macroscópicas. La otra cadena mamaria fue fijada e incluida en parafina. Sobre cortes de 5 μm, realizamos tinción de hematoxilina-eosina, ensayos de inmunohistoquímica y TUNEL. Evaluamos: proliferación y apoptosis celular; infiltración de mastocitos; expresión del REα y RP y de sus co-reguladores: SMRT, SRC-3; angiogénesis y factores pro-angiogénicos mediante área (AV) y densidad vascular (DV) y VEGF; porcentaje de conductos hiperplásicos y características del estroma circundante (densidad de núcleos del estroma). Por inmunofluorescencia se evaluó la co-localización de REα y sus co-reguladores. En las muestras de suero de animales post-puberales (DPN 50, DPN 110) medimos por RIA los niveles circulantes de $E_2$ y Pg.

**Resultados**

El tratamiento administrado a las madres (mediante mini-bombas osmóticas) no perturbó el normal desarrollo de la gestación, no se detectaron abortos y no se modificó la ganancia de peso durante la preñez. Después del parto, tanto las madres como las crías se mostraron saludables y en los parámetros evaluados no se diferenciaron de los controles.

La distancia ano-genital y el peso de las crías tampoco fueron modificados, sí existió un adelantamiento de la pubertad en las hembras de los grupos25 y 250 BPA. **ETAPA I:** La exposición *in utero* a BPA aumentó la relación proliferación/apoptosis tanto en el parénquima como en el estroma mamario en DPN 50, lo que indicaría una modificación del *turnover* celular. La desregulación del balance proliferación/apoptosis ocurrida en DPN 50, se manifestó con aumento en el porcentaje de conductos hiperplásicos (lesiones pre-neoplásicas) y en la densidad de núcleos del estroma, tanto en DPN 110 como en DPN 180. Todos los cambios se evidenciaron después de la pubertad, ya que en los animales de DPN 30, los parámetros evaluados no difieren entre hembras expuestas a BPA y a DMSO. En el grupo 250 BPA-DPN 50, los cambios en la histoarquitectura mamaria, se asociaron con aumento en la expresión de REα, disminución en la expresión de SRC-3, aumento en la angiogénesis y en la expresión de VEGF. Todos estos parámetros, excepto la expresión de VEGF, permanecieron modificados hasta DPN 110. En 250 BPA-DPN 110, describimos también una disminución en la expresión del co-regulador SMRT. La expresión del RP no fue diferente entre los grupos experimentales en ningún momento. La exposición *in utero* a BPA alteró transitoriamente la esteroideogénesis ovárica generando un ambiente endocrino con menores niveles de Pg en DPN 50. En DPN 110 y 180 importantes cambios en el estroma acompañaron a las lesiones pre-neoplásicas. Una capa densa de tejido conectivo rodeó a las estructuras epiteliales mamarias y un estroma fibroblástico reemplazó al tejido adiposo normal. Se observó además, un marcado aumento en la densidad de mastocitos asociados a las lesiones pre-neoplásicas. La presencia de este estroma fibroblástico acompañado de células inflamatorias se consideró un indicio de reacción desmoplásica. **ETAPA II:** Optimizamos el modelo de carcinogénesis con NMU en nuestra cepa Wistar, estableciendo que: a) la sensibilidad de la GM a la acción del NMU es mayor cuando se la administra en DPN 50 *vs* DPN 21; b) una dosis de 25 mg NMU/kg administrada en PND 50 puede considerarse sub-carcinogénica ya que no generó tumores en DPN 180 *vs* 83% de tumores con las dosis 50 mg NMU/kg. Confirmamos que la cepa Wistar puede ser considerada como de susceptibilidad media/alta frente a carcinógenos químicos. En DPN 110, los cambios en la histomorfología del parénquima y estroma mamarios en los grupos expuestos a BPA + 25 NMU fueron similares a los descriptos en los grupos expuestos solamente a BPA. Los cambios más notables se observaron en DPN 180, cuando la exposición *in utero* a 25 BPA aumentó la respuesta a la dosis sub-carcinogénica de NMU, el 13,3% de los animales (2/15) desarrollaron tumores de mama, comparado con 0/10 animales del grupo DMSO + 25 NMU. Todos los tumores

fueron encapsulados y de consistencia sólida con una marcada respuesta estromal caracterizada por fibrosis e infiltración mononuclear. La inmunotinción para CK8 y p63, permitió excluir invasión hacia el estroma por parte de las células epiteliales y la detección de REα y RP, ayudó a caracterizarlos como tumores hormonodependientes. Los tumores fueron clasificados como carcinomas ductales *in situ* con patrón papilar, cribiforme y mixto. En el grupo DMSO + 50 NMU, la incidencia tumoral fue del 70% (7/10 animales) y los tumores fueron clasificados como adenocarcinomas con patrón cribiforme, papilar y mixto. La multiplicidad tumoral fue similar cuando se comparó DMSO + 50 NMU (1,5 ± 0,8) *vs* 25 BPA + 25 NMU (2,5 ± 2,1).

**Conclusiones**

La exposición prenatal a BPA genera cambios en el parénquima y estroma mamario, evidentes mucho tiempo después que la exposición ha finalizado, considerados permisivos para el desarrollo de lesiones pre-neoplásicas/neoplásicas.

Dado que, la asociación entre la exposición prenatal a BPA y posnatal a una dosis sub-carcinogénica de un carcinógeno químico promovió la progresión de lesiones pre-neoplásicas a neoplasias podemos inferir que la exposición prenatal a BPA modula la respuesta de la GM a los carcinógenos químicos.

Los resultados obtenidos y presentados son consistentes con la hipótesis de que "la exposición *in utero* a BPA genera cambios histomorfológicos en la GM que pueden modificar la regulación endocrina de la misma y aumentar la susceptibilidad a carcinógenos químicos".

# *VIII- Abstract*

**Effects of Endocrine Disruptor Exposure on Mammary Gland Development and Function**

Endocrine disrupters (ED) are defined as "a substance or mixture of exogenous substances that alters one or more functions of the endocrine system and consequently cause adverse effects in the health of an intact organism, their linage or subpopulations". Some EDs mimic the actions of 17β-estradiol ($E_2$) and they have been named **xenoestrogens.** It has been hypothesized that in humans, xenoestrogen exposure could increase the incidence of hormo-dependent cancer. One of the most studied xenoestrogen is bisphenol A (BPA), used in the preparation of epoxy resins, the manufacture of polycarbonate plastics and, as a component of dental sealants among others. The human risk to be exposed to BPA is potentially high due to the daily use of products that contain this chemical (plastics packages of beverages and food, baby bottles, compact discs, laquers and paintings) and because BPA leaches from the containers with high temperatures, strong alkalis or acids. In our laboratory, we are evaluating the effects of perinatal exposure to BPA, not only in laboratory rodents but in farm animals and wildlife species.

The mammary gland (MG) is the organ which defines mammals and plays a main role in offspring nutrition. The MG development begins *in utero* and ends in the adulthood. Several hormones and growth factors regulate the mammary development. MG is a hormo-dependent organ. The rat possesses six ventrolateral pairs of MG, bilaterally arranged, from the cervical to the inguinal regions. The rat MG parenchyma consists of a network of ducts which convey milk from alveoli (the place of milk synthesis) to nipple. The main or primary ducts grow by branching and small ducts or ductules arise. Terminal end buds (TEBs); terminal ducts (TDs) or blunt end buds and alveolar buds (ABs) are immature epithelial structures. The epithelium is embedded in a sheath of connective tissue stroma, surrounded by adipose tissue (fat pad), that is limited by a connective tissue capsule. The development of the mammary ductal tree depends on stromal–epithelial interactions, and these interactions are important in embryonic and postnatal development.

Ovarian steroids (estrogens and progesterone -P-) are known regulators of cellular growth and differentiation in several organs and tissues, acting through its receptors: estrogen receptor (ER) and progesterone receptor (PR), in combination with transcriptional factors and co-regulators (co-activators and co-repressors). Estrogens are powerful mitogens in the MG, necessary as much for the normal development as for the induction

and progression of mammary carcinomas. The signaling pathway of P-PR is implied in lobuloalveolar branching and growth of the MG.

In the last decades, epidemiological studies showed a high incidence of hormo-dependent tumors and especially of breast cancer which is concomitant with the increased presence of EDs in the environment.

There are three theories related to the onset of cancer: 1) the **somatic mutation theory**; 2) the **epigenetic theory**; 3) the **tissue organization field theory**. The genetic changes that support carcinogenesis alter main properties of the cells and allow them to evade normal growing controls and finally, acquire the phenotype of a cancerous cell. The epigenetic and tissue organization could better explain the relationship between ED and cancer. In the carcinogenic process there are two main components: the carcinogen and the target tissue. Several substances or agents produce genetic injury and promote that cells become malignant. Breast cancer is the most common form of cancer among women. Exposure to estrogens during lifetime is a main risk factor for the development of breast cancer. Epidemiological studies suggest a strong correlation between exposure to estrogens/xenoestrogens and cancer, numerous experimental studies done both *in vivo* and *in vitro* support this issue. Different animal models were used to study the biology of cancer and for therapeutic and prevention strategies development. The two most used chemical-carcinogenesis models are those that employ DMBA (7,12-dimethylbenz(α)anthracene) and NMU (*N*-methyl-*N*-nitrosourea). There is a correlation between changes induced by carcinogens and the structure of MG present at the time of drug administration. The susceptibility of MG is maximal when the carcinogen is administered at 45-60 days of age, which coincides with the period when TEBs are actively differentiating into ABs. Once the carcinogen is administered, the TEBs in the gland undergo a transformation towards intraductal proliferations or hyperplastic ducts, named preneoplastic lesions. This is the first step in the process of tumorigenesis, then the lesions can progress to ductal carcinoma *in situ* and to adenocarcinoma. Mammary stroma modifications, mast cells infiltration and neovascularization have been linked to tumor progression process.

Our hypothesis proposes that exposure to low doses of a xenoestrogen during critical periods of histiogenesis/organogesis/differentiation of the MG, modifies either the balance between proliferation and apoptosis and /or the tissue microenvironment promoting the action of chemical carcinogens.

**Objectives**

In Wistar rats,

**PART I:** To study the effect of *in utero* exposure to low doses of BPA on the MG of female offspring. To achieve this aim the following parameters were evaluated:

- Body weight gain and the onset of puberty;
- Histoarchitecture of MG parenchyma and stroma,
- Immunocompetent cells infiltration in the MG
- MG endocrine microenvironment;
- MG angiogenesis

**PART II:** To examine whether *in utero* exposure to BPA increases its susceptibility to the carcinogen *N*-nitroso-*N*methylurea (NMU). To achieve this aim we assessed:

- MG sensitivity to a chemical carcinogen;
- Changes in the histomorphological features of the MG
- Incidence and multiplicity of mammary tumors and pre-neoplastic lesions
- Tumor histomorphology and hormone-dependence.

**Materials and Methods**

Wistar rats received through sc osmotic pumps, from day 8 of gestation (GD8) up to the delivery: vehicle (dimethyl sulfoxide, DMSO) or BPA: 25 μg/kg bw/d; 250 μg/kg bw/d or 25 mg/kg/d (25 BPA, 250 BPA and 25 mBPA, respectively). Only female offspring were used in this study. **PART I:** Ano-genital distance was evaluated in newborn. The body weight was recorded from birth to 110 days of age; vaginal opening was registered as the beginning of the puberty. Animals were sacrificed at 30, 50, 110 or 180 days of age, considered postnatal day (PND: 30, 50, 110, 180). **PART II:** MG susceptibility to carcinogen NMU was evaluated in two pilot experiments. First, by injecting female rats in PND 21 (before the puberty) or PND 50 (after puberty). Second, testing carcinogenic effect of 25 NMU taking as reference the tumor incidence in rats receiving 50 NMU (50 mg/kg bw) a dose previously defined as carcinogenic. Once the model was optimized, animals, prenatally exposed to BPA or DMSO were injected with a 25 NMU at PND 50 and sacrificed at PND 110 or PND 180. To confirm NMU carcinogenic activity and rat strain susceptibility, a third group of females exposed *in utero* to DMSO, was injected with 50 NMU (positive control) and sacrificed on PND 180.

Two hours before sacrifice all animals received bromodeoxyuridine. Serum samples and abdomino-inguinal MG chains were obtained.

One mammary chain was processed as whole mount to detect micro and/or macroscopic lesions. The other was fixed and embedded in paraffin. Serial 5-μm paraffin sections were stained with hematoxylin-eosin or immunostained. We evaluated: cellular proliferation and apoptosis, mast cells infiltration, expression of ERα, PR, SMRT, SRC-3, angiogenic process and pro-angiogenic factors, percentage of hyperplastic ducts and stromal nuclei density. Co-expression of ERa and co-regulators was evaluated using immunofluorescence. Circulating levels of $E_2$ and P were measured by RIA at PND 50 and PND 110.

**Results**

The treatment administered to the dams (through osmotic pumps) did not produce miscarriage; neither disturbs the normal development of gestation nor the body weight gain of the dams. **PART I**: The ano-genital distance and the mean body weight of lactating offspring were not modified. Female offspring exposed *in utero* to BPA exhibited advanced puberty, measured as early vaginal opening compared to controls. BPA exposure increased the proliferation/apoptosis ratio in both the mammary parenchyma and stroma on PND 50, suggesting an alteration of cellular turnover. The impaired balance between proliferation and apoptosis on PND 50, was correlated with increased percentage of hyperplastic ducts (pre-neoplastic lesions) and the stromal nuclei density on PND 110 and PND 180. All changes in the mammary gland occurred after puberty, since in females at PND 30, the parameters evaluated were not different between BPA and DMSO groups. On PND 50, 250 BPA exposed females exhibited alterations in mammary histoarchitecture that were associated with higher expression of ERα, lower expression of SRC-3, increased angiogenesis and high VEGF expression. Except for the increased expression of VEGF, all changes remained until PND 110. Moreover, at PND 110, in 250 BPA group the expression of SMRT was lower than the control group animals. The expression of PR was not modified in any experimental group. Serum level of P showed a significant reduction in 25 BPA and 250 BPA groups. *In utero* exposure to BPA transitory alters the ovarian steroidogenic providing and endocrine milieu with lower levels of P on PND 50. On PND 110 and PND 180, the stroma associated with hyperplastic ducts showed signs of desmoplasia and contained an increased number of mast cells. **PART II**: Results from pilot experiments stated that, in our Wistar strain: a) the MG sensitivity to NMU action is

higher when the administration occurs on PND 50 compared with PND 21; and b) since, at PND 180 mammary tumor incidence after NMU administration (at PND 50) were 0% for the group receiving 25 NMU and 83% for 50 NMU, 25 NMU can be considered a subcarcinogenic dose. We confirmed that Wistar strain has medium/high sensitivity to NMU. On PND 110, the changes in the mammary parenchyma and stroma in BPA + 25 NMU groups were similar to those described to the groups only exposed to BPA. At PND 180, *in utero* exposure to 25 BPA, enhanced the response to a subcarcinogenic NMU dose, results were as follow: 13.3% (2/15) of animals developed mammary malignancies compared with 0/10 animals in the DMSO + 25 NMU group. All tumors were encapsulated and of solid consistency, and the stromal response demonstrated by fibrosis and mononuclear infiltration was a common feature. CK8 and p63 immunostaining patterns ruled out stromal invasion by epithelial cells. All tumors were ERα and PR positive, thus they could be classified as hormone-dependent tumors. Tumors were classified as ductal carcinoma *in situ* with cribriform, papillary, or mixed pattern (cribriform and papillary). Mammary tumors incidence in DMSO + 50 NMU was 70% (7 of 10) and were classified as adenocarcinoma of papillary, cribriform, or mixed. Tumor multiplicity did not differ between DMSO + 50 NMU and 25 BPA + 25 NMU (1.5 ± 0.8 *versus* 2.5 ± 2.1 respectively).

**Conclusions**

Our results demonstrate that the prenatal exposure to low doses of BPA perturbs mammary gland histoarchitecture and increases the carcinogenic susceptibility to a chemical challenge administered 50 days after BPA exposure ended.

The increased angiogenesis, the fibrotic response in the stroma and the increased mast cell number spatially associated with hyperplastic ducts, observed in the mammary glands of adult animals exposed prenatally to BPA may play a permissive, if not cocausal, role regarding NMU-induced carcinogenesis.

Results presented in this thesis are consistent with our hypthesis that “the *in utero* exposure to BPA generates histomorphological changes in the MG that could modify the its endocrine regulation and increase its susceptibility to a chemical carcinogen”.

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