



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Câmpus de São José do Rio Preto

Ellen Cristina Rivas Leonel

**Efeitos da exposição neonatal ao bisfenol A e ao 17- β estradiol
sobre a glândula mamária de fêmeas adultas de gerbilo da
Mongólia**

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto.

Financiadora: FAPESP – Processos 2015/01548-5, 2016/22947-8 – e CAPES.

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RESUMO

A incidência de câncer de mama tem aumentado consideravelmente ao longo dos anos. Frente a esta alta taxa epidemiológica a atenção às causas desta doença tem sido significativa em meio a grupos de pesquisa. Fatores genéticos e algumas situações de risco, como a exposição a componentes químicos disruptores endócrinos, podem estimular o seu desenvolvimento. O bisfenol A (BPA), utilizado na manufatura de plásticos e resinas, pode ser liberado destes componentes e exercer efeitos estrogênicos, causando distúrbios na função endócrina e aumentando a predisposição à carcinogênese. Alguns estudos têm demonstrado os efeitos do BPA em longo prazo, de modo que sua exposição durante o período de desenvolvimento embrionário inicial e perinatal pode predispor a glândula mamária (GM) ao desenvolvimento de câncer em indivíduos já adultos. Seu mecanismo de ação, assim como o do estradiol endógeno, baseia-se principalmente na ligação a receptores celulares hormonais, podendo estimular a proliferação, promovendo um desbalanço nos ciclos de renovação celular mamária. Para o estudo de carcinogênese o gerbilo da Mongólia (*Meriones unguiculatus*) é um modelo experimental interessante, já que possui capacidade de desenvolver tumores espontâneos em órgãos hormônio-responsivos, mimetizando, naturalmente, o ambiente de um indivíduo predisposto à tumorigênese. Assim, os objetivos deste trabalho foram (1) caracterizar morfológicamente a GM de fêmeas de gerbilo da Mongólia em diferentes estágios funcionais, (2) descrever as alterações morfológicas encontradas na GM de fêmeas adultas expostas ao BPA e a 17- β estradiol durante o período de desenvolvimento perinatal avaliando a presença de lesões histopatológicas, índice de proliferação celular e expressão do marcador de alteração epigenética, EZH2, bem como (3) descrever as variações na expressão de receptores levando em consideração níveis séricos hormonais, com o intuito de esclarecer os possíveis efeitos do BPA administrado durante o desenvolvimento embrionário na GM adulta. Nossos resultados demonstraram que aos 6 meses de idade houve um aumento significativo no índice proliferativo, na incidência de hiperplasia e considerável alteração na deposição de fibras colágenas no compartimento estromal da glândula. Verificou-se aumento na expressão de EZH2 nas células epiteliais mamárias dos animais do grupo tratado apenas com o óleo de milho (veículo), revelando uma ação inesperada do mesmo na GM. As análises de receptores hormonais mostraram que diferenças significativas nas taxas de expressão ocorreram nas GMs dos grupos tratados tanto com BPA quanto com 17- β estradiol em comparação ao grupo controle intacto. Com isso, pôde-se concluir que a exposição neonatal ao BPA causa alterações morfológicas e moleculares na GM de gerbilos da Mongólia refletidas após 6 meses de idade, que podem indicar sinais de pré-malignidade, confirmando o efeito carcinogênico deste composto.

Palavras-chave: gerbilo da Mongólia, receptor hormonal, disruptor endócrino, morfologia, histopatologia.

ABSTRACT

The incidence of breast cancer has increased considerably over the years. Facing this high epidemiological rate, attention to the causes of this disease has been significant among research groups. Genetic factors and some risk situations, such as exposure to chemical endocrine disrupting components may stimulate its development. Bisphenol A (BPA), a compound used in the manufacture of plastics and resins, can be released from these components and exert estrogenic effects, causing disturbances of endocrine function and increasing the predisposition to carcinogenesis. Some studies have demonstrated the long-term effects of BPA, in a way that exposure during embryonic development (perinatal phase) may predispose the mammary gland (MG) to development of cancer in adults. Its mechanism of action, as that of endogenous estradiol, is mainly based on cellular hormonal receptors binding, which can stimulate the proliferation, promoting an imbalance of breast cell cycles and renewal. For these carcinogenesis studies, the Mongolian gerbil (*Meriones unguiculatus*) is an interesting experimental model, since it has the ability to develop spontaneous tumors in hormone-responsive organs, naturally mimicking the environment of an individual predisposed to tumorigenesis. Thus, the aims of this study were to (1) characterize morphologically the mammary gland of Mongolian gerbil females at different stages of functionality, (2) describe morphological changes found in the MG of adult females exposed to BPA or 17- β estradiol during the perinatal development evaluating the presence of histopathological lesions, cell proliferation index and expression of the epigenetic alteration marker, EZH2, as well as (3) to describe the variations in hormone receptors expression taking into account hormonal serum levels, with the purpose of clarifying the possible effects of BPA administered during embryonic development in adult MG. Our results demonstrated that at 6 months age there was a significant increase in proliferative index, incidence of hyperplasia and considerable alteration in the deposition of collagen fibers in the stromal compartment of the gland. There was an increase in EZH2 expression in the mammary epithelial cells of animals treated with corn oil (vehicle), revealing an unexpected action of it in MG. Hormone receptor analysis showed that significant differences in expression rates occurred in the mammary glands of the groups treated with both, BPA and 17 β -estradiol compared to the intact control group. Thus, we observed that neonatal exposure to BPA causes morphological and molecular changes in the MG of Mongolian gerbils reflected after 6 months age, which may indicate signs of pre-malignancy, confirming the carcinogenic effect of this compound.

Keywords: Mongolian gerbil, hormonal receptor, endocrine disruptor, morphology, histopathology.

LISTA DE ABREVIATURAS E SIGLAS

AHR	Receptor de aryl hidrocarbono
AKT	Proteína quinase
BMP	Proteína morfogenética óssea
BPA	Bisfenol A
CPG	Região citosina fosfato guanina
DE	Disruptor endócrino
ERα	Receptor de estrógeno α
ERβ	Receptor de estrógeno β
EZH2	Enzima histona-lisina metiltransferase (<i>enhancer of zeste homolog 2</i>)
FGF	Fator de crescimento de fibroblastos
FSH	Hormônio folículo estimulante
GM	Glândula mamária
HPLC	Cromatografia líquida de alta eficiência
JAK2	Janus quinase 2
LH	Hormônio luteinizante
MAPK/ERK	Proteino-quinases ativadas por mitógenos (<i>mitogen-activated protein kinases</i>)
MB	Membrana basal
MEC	Matriz extracelular
mRNA	RNA mensageiro
PI3K/AKT	Via da fosfatidil inositol 3-quinase / proteína quinase B
PR	Receptor de progesterona
PRLR	Receptor de prolactina
PTHrP	Peptídeo relacionado ao hormônio da paratireoide
RANK-L	Ligante do receptor ativador do fator nuclear kappa B

STAT	Sinais transdutores e ativadores de transcrição
TEB	Broto terminal (<i>terminal end bud</i>)
TNF	Fator de necrose tumoral
WNT	Via de sinalização “wingless/integrated”

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1 INTRODUÇÃO

De acordo com a GLOBOCAN 2018, estima-se que em 2018, 18,1 milhões de novos casos de câncer tenham ocorrido mundialmente (BRAY et al., 2018) e acredita-se que o índice anual deva ainda subir para 23,6 milhões até 2030 (SIERRA et al., 2016). Frente à alta taxa epidemiológica, a atenção às causas desta doença tem sido significativa em meio a grupos de pesquisa. Já foi sugerido que a maioria dos novos casos que surgem são devido a distúrbios hormonais, reprodutivos ou relacionados ao consumo de alimentos (BRAY et al., 2016).

O câncer de mama compreende 1 em cada 4 casos de câncer em mulheres no mundo e é o câncer com a maior taxa de mortalidade na grande maioria dos países (BRAY et al., 2018). Apesar do conhecimento sobre sua etiologia ser limitado, fatores genéticos e algumas situações de risco podem estar relacionados ao estímulo do desenvolvimento da doença (HOWELL et al., 2014). Como exemplo pode-se mencionar a conhecida relação entre o risco de desenvolvimento de câncer de mama e altos níveis séricos de compostos químicos disruptores endócrinos (DE) (PASTOR-BARRIUSO et al., 2016). Um exemplo de DE bastante conhecido e estudado é o bisphenol A (BPA), um composto químico utilizado na manufatura de plásticos policarbonatos, resinas epóxi, poliésteres e outros componentes (STAPLES et al., 1998). A partir destes produtos manufaturados, BPA é liberado e entra em contato com os organismos através do ar, da água e dos alimentos (MICHAŁOWICZ, 2014). Uma vez em contato com as células pode exercer efeitos estrogênicos, causando distúrbios na função do sistema endócrino (VANDENBERG et al., 2007a) e, conseqüentemente, aumentando a predisposição à carcinogênese (KERI et al., 2007).

Frente à possibilidade da ocorrência de efeitos causados em longo prazo pelo BPA, interesse tem sido dado à possibilidade de o mesmo causar no indivíduo adulto alterações neoplásicas após exposição prematura ao composto. Por exemplo, em animais expostos ao BPA durante o período perinatal há predisposição ao câncer de glândula mamária (GM), cuja causa é comprovadamente relacionada ao prévio contato com o composto (DOHERTY et al., 2010).

A alta eficácia em proporcionar maleabilidade e auxiliar na laminação de resinas epóxi durante a fabricação de plásticos (FRANK; KEENAN; PLANT, 1967) bem como o reduzido valor de mercado de produtos manufaturados contendo altas

concentrações de BPA, são fatores que estimularam o uso indiscriminado deste composto. Entretanto, alguns países já proibiram o uso de BPA na composição de mamadeiras (MICHAŁOWICZ, 2014) e sua dose de referência máxima segura estabelecida pela *US Environmental Protection Agency* (US EPA, 2012) foi de 50 µg/kg/dia.

1.1 MORFOLOGIA, FISILOGIA E EMBRIOLOGIA DA GLÂNDULA MAMÁRIA

O conhecimento sobre morfologia, fisiologia e patologia mamária é essencial para o desenvolvimento de novos tratamentos contra diversas doenças relacionadas à glândula. A GM é classificada como exócrina, uma vez que forma ductos que conduzem a secreção para os mamilos; é funcionalmente classificada como apócrina, já que os lipídeos do leite são liberados juntamente com porções do citoplasma apical das células epiteliais, bem como merócrina, já que as proteínas do leite são liberadas por exocitose (VERMA; AMBATIPUDI, 2016). É composta por diversos lóbulos de glândulas túbulo-alveolares compostas, cujo número pode variar de acordo com a espécie em questão. Cada lóbulo consiste de uma unidade secretora da glândula (JUNQUEIRA; CARNEIRO, 2017).

É importante esclarecer que há diferenças entre as espécies com relação ao desenvolvimento, morfologia e fisiologia da GM, de modo que a presente revisão de literatura irá abordar informações referentes à GM de roedores.

A título de nomenclatura, as estruturas que compõem a GM são: (i) ductos, estruturas tubulares que já estão presentes no momento do nascimento; (ii) brotos terminais, conhecidos como *terminal end buds* (TEB); (iii) brotos alveolares e (iv) alvéolos maduros (HENNIGHAUSEN; ROBINSON, 2005). Estas estruturas compõem a porção parenquimatosa da GM, que limita-se, em meio ao estroma, pela presença de uma membrana basal (MB) que é uma especialização da matriz extracelular (MEC) (KIMATA et al., 1985). Cada uma destas estruturas será discutida em detalhes a seguir.

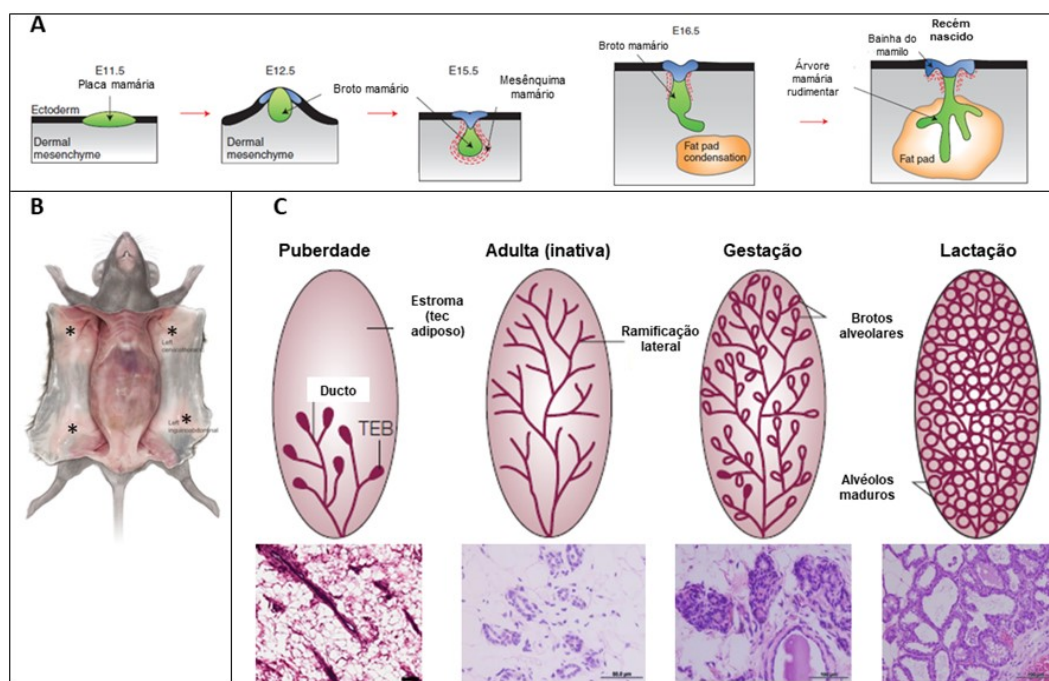
Com relação à origem embrionária da GM, sua porção parenquimatosa deriva do ectoderma, e seu estroma, do mesoderma (IMAGAWA; BANDYOPADHYAY; NANDI, 1990). O início de seu desenvolvimento se dá durante o período embrionário.

A GM é uma glândula sudorípara modificada que se origina como uma invaginação do epitélio de revestimento em direção ao conjuntivo. A formação de cada uma das glândulas inicia-se devido a uma invaginação de um grupo de células do ectoderma, que ocorre por volta dos 10 a 11 dias de idade embrionária em ratos e camundongos (MEDINA, 1996; RICHERT et al., 2000). O mesênquima se condensa e a epiderme torna-se espessa. O epitélio vai dando origem à placa mamária e, no mesênquima subjacente, papilas mamárias secundárias vão sendo formadas para tornarem-se ductos lactíferos que se abrem para a superfície dos tetos em desenvolvimento. Os tetos, por sua vez, são formados por proliferação do mesênquima, que eleva a epiderme ao redor das aberturas glandulares (HYTTEL; SINOWATZ; VEJLSTED, 2012).

A localização destas invaginações segue duas linhas laterais ventrais no embrião em desenvolvimento, denominadas cristas mamárias. Longitudinalmente ao longo do sulco mamário, as glândulas primitivas vão se desenvolvendo, resultando em várias papilas com aberturas (HYTTEL; SINOWATZ; VEJLSTED, 2012). Após a formação deste broto primário, por volta do 16º dia embrionário este epitélio começa sua progressão em direção ao estroma mesodérmico, ramificando-se até formar ductos secundários revestidos por um epitélio simples cúbico (Figura 1). Ao nascimento estão presentes apenas 2 a 4 ramificações ductais, que apresentarão crescimento não significativo até a puberdade (HENNIGHAUSEN; ROBINSON, 2005; MEDINA, 1996).

O período de desenvolvimento mamário pré-natal é dependente de hormônio e regulado por cascatas de sinalização entre o epitélio e o mesênquima. Vias de sinalização de etapas específicas do desenvolvimento embrionário têm sido caracterizadas em camundongos (ROBINSON, 2004). Inicialmente, assim que o broto se forma, um halo de células mesenquimais surge ao redor do epitélio invaginado, permanecendo próximo à epiderme e auxiliando na formação do mamilo (ROBINSON, 2004).

Figura 1. Desenvolvimento embrionário e pós-natal da glândula mamária de roedores. (A): visão geral do desenvolvimento mamário embrionário dos dias de desenvolvimento embrionário 11,5 (E11.5) a 16,5 (E16.5). Adaptado de Cowin & Wysolmerski (2010) (COWIN; WYSOLMERSKI, 2010a). (B): Esquema mostrando a distribuição dos quatro pares de glândulas mamárias de roedores (*). Adaptado de Cardiff et al (2018) (CARDIFF et al., 2018). (C): apresentação esquemática e histológica dos diferentes estágios de desenvolvimento mamário pós-natal: estrutura ductal rudimentar que se faz presente até a puberdade, quando a secreção de estrógeno e progesterona promovem o crescimento ductal. Nos animais em idade reprodutiva, a cada ciclo estral ramificações laterais se desenvolvem e, posteriormente envolvem. Secreção de prolactina, hormônios placentários e progesterona estimulam a formação de brotos alveolares, que se multiplicam e crescem durante a lactação para promover a secreção láctea. Adaptado de LaRocca et al (2011) (LAROCCA; PIETRUSKA; HIXON, 2011), Hennighausen & Robson (2005) (HENNIGHAUSEN; ROBINSON, 2005) e Leonel et al 2017 (LEONEL et al., 2017).



Sabe-se que a via de sinalização WNT está diretamente relacionada com o início da formação mamária, sendo um fator chave em sua indução (DASGUPTA; FUCHS, 1999). Isso leva a crer que células ectodérmicas são recrutadas para as placas mamárias via sinalização WNT (VELTMAAT et al., 2003). Ainda, camundongos knockout *K14-Dkk1* (com ausência do gene Dickkopf-1, um inibidor da via WNT) não apresentam brotos mamários, confirmando a ação indispensável desta via na formação mamária (ANDL et al., 2002). Sinalização por fator de

crescimento de fibroblastos (FGF) também parece exercer forte influência na interação epitélio/estroma no momento do crescimento e direcionamento dos brotos, dada a expressão de receptores FGF 2-IIIb (SPENCER-DENE et al., 2001) e FGF10 (presente no tecido adiposo a partir do 15º dia de desenvolvimento embrionário em camundongos) que, quando ausente resulta no bloqueio da indução de algumas placas mamárias (MAILLEUX et al., 2002). Outras vias de sinalização estão presentes nas primeiras etapas do desenvolvimento mamário e são mencionadas na Tabela 1.

Tabela 1. Vias de sinalização atuantes nas primeiras etapas do desenvolvimento mamário embrionário em camundongos.

Via	Local de ação	Momento da ação	Referência
WNT	Indução das placas	10º DE	(DASGUPTA; FUCHS, 1999)
FGF	Placas mamárias	11º DE	(MAILLEUX et al., 2002)
Hedgehog (HH)	Diferenciação de células da placa	NE	(VELTMAAT et al., 2003)
<i>Msx1</i> e <i>Msx2</i>	Invaginação do broto	12º DE	(SATOKATA et al., 2000)
BMP	Mesênquima e epitélio	12º DE	(PHIPPARD et al., 1996)
PTHRP	Epitélio do broto e tecido adiposo	11º-18º DE	(DUNBAR; WYSOLMERSKI, 1999)

DE: dia de desenvolvimento embrionário, NE: não especificado; BMP: proteína morfogenética óssea; PTHRP: peptídeo relacionado ao hormônio da paratireoide.

Com relação à ação hormonal neste período do desenvolvimento, alguns autores afirmam não haver influência sistêmica (endócrina) uma vez que as diferenciações desta fase ocorrem normalmente *in vitro* sem ação suplementar hormonal ou de fatores de crescimento (HENNIGHAUSEN; ROBINSON, 2001; HOVEY; TROTT; VONDERHAAR, 2002; KRATOCHWIL, 1969). Entretanto há evidências da ação do estradiol na GM embrionária devido à presença de receptores de estrógeno (ER α e ER β) no mesênquima ao redor do broto (LEMMEN et al., 1999), tendo Wadia et al. (2013) já documentado a expressão exclusiva de mRNA de ER α no compartimento estromal (WADIA et al., 2013). Com relação à

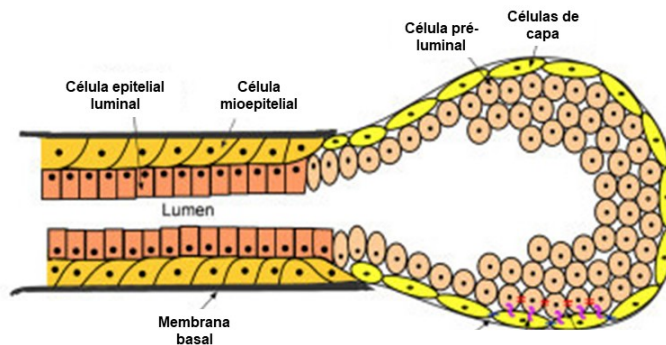
progesterona sabe-se que sua ação é necessária apenas na proliferação ductal que ocorre na gestação (CONNEELY; JERICEVIC; LYDON, 2003). Alta exposição fetal a hormônios maternos pode ainda, muitas vezes, ocasionar expressão de proteínas do leite em recém-nascidos (MACIAS; HINCK, 2012).

Entre o nascimento e a puberdade, a glândula sofre um crescimento alométrico, acompanhando o crescimento corporal; sob ação dos hormônios ovarianos na puberdade, ocorre proliferação expansiva, de modo que os ductos crescem em direção ao tecido adiposo (MACIAS; HINCK, 2012).

Ao atingir a puberdade as porções terminais dos ductos são dotadas de células com elevada taxa mitótica – as TEBs –, que proliferam promovendo o crescimento ductal em meio ao tecido adiposo mamário (HUMPHREYS et al., 1996). TEBs constituem, portanto, estruturas temporárias altamente responsivas ao estímulo de esteróides secretados pelos ovários (HENNIGHAUSEN; ROBINSON, 2005). Estes brotos são compostos por células do corpo da TEB, ou células luminais, bem como células de capa da TEB (Figura 2). Estas últimas encontram-se na camada mais externa e se comunicam com o estroma através de uma MB (HUMPHREYS et al., 1996); acredita-se que sejam progenitoras das células mioepiteliais uma vez que expressam miosina (DULBECCO et al., 1983).

A MB, composta por uma especialização da MEC, estabelece contato entre o parênquima e as células basais em todos os estágios de desenvolvimento da GM. Esta é possivelmente responsável pela regulação do crescimento e diferenciação das células secretórias luminais e impacta na relação epitélio estroma durante o desenvolvimento (FARALDO et al., 2006). A MB, que limita o epitélio é basicamente composta por colágeno tipo IV, secretado pelas células epiteliais (LIOTTA et al., 1979), além de outras proteínas como laminina e proteoglicanos. A ausência deste tipo de colágeno em modelos de cultivo 3D prejudica as interações epitélio-estroma bem como proliferação e desenvolvimento do epitélio mamário (WICHA et al., 1980). Isto leva a concluir que a presença de uma MB, além de indispensável para a organização estrutural da GM, também possui papel fundamental em sua fisiologia.

Figura 2. Esquemática da estrutura da TEB. Adaptado de Manivannan & Nelson, 2012 (MANIVANNAN; NELSON, 2012).



Com a puberdade, picos de secreção de estrógeno estimulam um acúmulo de tecido conjuntivo denso e tecido adiposo entre os lóbulos secretores (HYTTEL; SINOWATZ; VEJLSTED, 2012). Os ciclos estrais proporcionam alterações frequentes na morfologia do epitélio mamário de roedores, de modo que a secreção de estrógenos durante o proestro estimula a síntese de DNA, aumentando a taxa mitótica durante o estro e o metaestro (GRAHAME; BERTALANFFY, 1972). Este crescimento é seguido de uma regressão que ocorre na fase luteínica (SINHA; TUCKER, 1966). À medida que os ciclos estrais vão se repetindo, a estrutura dos ductos e alvéolos vai sendo estabelecida nas GMs (HYTTEL; SINOWATZ; VEJLSTED, 2012).

Já o desenvolvimento funcional e estrutural se conclui quando a fêmea fica prenhe e dá à luz (HYTTEL; SINOWATZ; VEJLSTED, 2012). Durante a gestação, corpo lúteo e placenta secretam progesterona e estrógeno que irão proporcionar o aumento dos lóbulos secretores. Sabe-se que estes dois hormônios exercem um efeito supressor na ação da prolactina, hormônio responsável pelo desenvolvimento da GM, produção e secreção dos constituintes proteicos e não proteicos do leite. Com a progressão da gestação, o corpo lúteo começa a involuir e a secreção destes hormônios reduz, promovendo o aumento da atividade da prolactina (ROSS; PAWLINA, 2007).

O desenvolvimento mamário gestacional conta com um considerável crescimento ductal por proliferação das células epiteliais, que tomam o lugar do tecido adiposo para estabelecer a real estrutura lóbulo-alveolar da GM (KNIGHT; PEAKER, 1982). A relação epitélio/estroma durante a gestação cresce

consideravelmente em camundongos (RICHERT et al., 2000) bem como em gerbilo da Mongólia (*Meriones unguiculatus*) (LEONEL et al., 2017). Ao final da lactação, as estruturas alveolares ocupam praticamente toda a extensão mamária, anteriormente ocupada por tecido adiposo. Neste momento, os alvéolos começam a apresentar secreção láctea, contendo proteínas e lipídeos (RICHERT et al., 2000). A expressão de α -actina ao redor dos ácinos apresenta um aumento considerável já nos primeiros estágios da gestação em ratas (HUA et al., 2000) e em gerbilos (LEONEL et al., 2017). Este aumento alveolar é independente de estrógeno, visto que há uma queda considerável na expressão de ER- α em células epiteliais mamárias nesta fase (LEONEL et al., 2017). Receptores de progesterona (PR), pelo contrário, são essenciais durante o crescimento lóbulo-alveolar. Sabe-se que as células que expressam PR exercem um efeito proliferativo parácrino nos ácinos, visto que elas localizam-se sempre ao lado de células em proliferação (HENNIGHAUSEN; ROBINSON, 2005). Tal efeito pode estar relacionado à ação da molécula RANK-L, que pertence à família TNF (MULAC-JERICEVIC et al., 2003).

Após o parto, a queda drástica na produção de estrógeno e progesterona permite que a prolactina possa desenvolver seu papel lactogênico (ROSS; PAWLINA, 2007). Durante a lactação este hormônio tem função essencial e sua secreção pelo epitélio mamário, em adição àquela da adeno-hipófise, também já foi descrita (VONDERHAAR, 1999). Ele age por meio do receptor transmembrana PRLR, que quando ligado ao hormônio permite a ativação de STAT5 e JAK2 (HENNIGHAUSEN; ROBINSON, 2005), proteínas que regulam funções celulares como proliferação, diferenciação e sobrevivência (RANI; MURPHY, 2016), fatores essenciais na GM durante gestação e lactação. Pode-se afirmar, portanto, que a cascata de ativação PRLR, STAT5 e JAK2 é essencial para o processo de proliferação e diferenciação das células luminiais mamárias durante a lactação.

A ejeção do leite, por sua vez, é um fenômeno dependente de estímulo mecânico nos mamilos durante a sucção pelo filhote. Com isso, impulsos são enviados a partir dos mecanorreceptores nos mamilos para o hipotálamo, promovendo a secreção de prolactina pela adeno-hipófise. Os mecanorreceptores são responsáveis também pela secreção de ocitocina pela neuro-hipófise, que promove a contração das células mioepiteliais (ROSS; PAWLINA, 2007).

Durante o período de produção láctea da GM o produto secretado pelos lóbulos é liberado em um ducto excretor, denominado ducto galactóforo

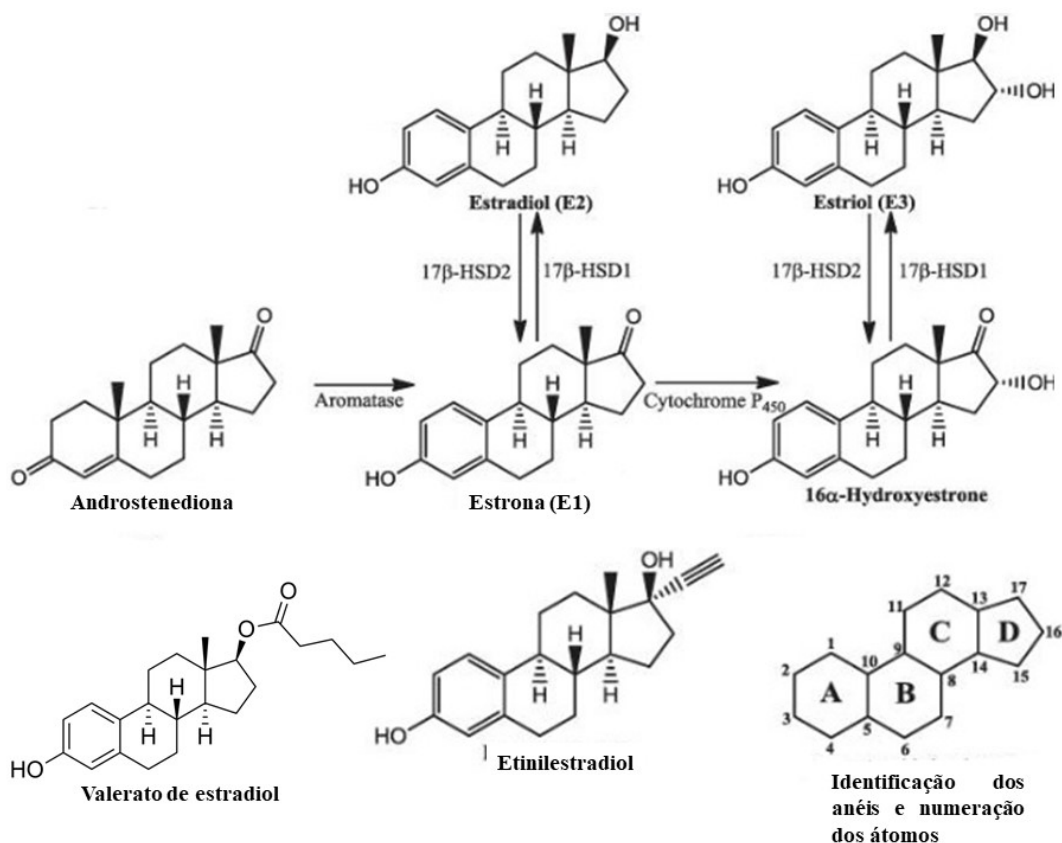
(JUNQUEIRA; CARNEIRO, 2017). Este é caracterizado pela presença de um epitélio contendo uma ou duas camadas de células cuboidais epiteliais (ROSS; PAWLINA, 2007). O número de ductos galactóforos varia entre as espécies. Sabe-se que em roedores da subordem *Myomorpha* cada teto apresenta apenas um ducto galactóforo, ao passo que aqueles pertencentes à subordem *Hystricomorpha* podem apresentar até 8 ductos em cada teto (YU; ANDERSON, 1975). O ducto galactóforo emerge independentemente no mamilo. Já na região mais próxima à superfície do mamilo, os ductos se modificam em um epitélio estratificado pavimentoso queratinizado (ROSS; PAWLINA, 2007).

A regressão mamária após o fim do estímulo mecânico das mamadas se dá pela remoção de células secretoras e remodelamento da árvore ductal, com a involução da glândula que retorna à sua estrutura prévia (MACIAS; HINCK, 2012). Duas fases da involução glandular foram descritas. A primeira fase, até 12 horas após o fim das mamadas, é reversível com o retorno das mesmas e é caracterizada pela ocorrência de apoptose e destacamento de células epiteliais, com acúmulo de células no lúmen. Já a segunda fase é irreversível e ocorre a partir de 48 horas após o fim das mamadas, apresentando desestruturação dos alvéolos além de desnaturação da MEC. Após o completo remodelamento o tecido é novamente substituído por adipócitos então o tecido adiposo reassume como o principal constituinte da glândula (MACIAS; HINCK, 2012). A família de proteínas STAT também apresenta papel importante na transição de involução mamária, via sinalização AKT. Durante a primeira fase da involução a ativação de STAT3 antagoniza a ação pró-sobrevivência de STAT5 (LI et al., 1997), induzindo vias apoptóticas (CLARKSON et al., 2006) bem como promovendo a liberação de enzimas lisossômicas que promovem morte celular inicial sem envolver vias apoptóticas clássicas (KREUZALER et al., 2011). A segunda fase de involução conta com fatores sistêmicos, como o plasminogênio sintetizado no fígado, e ação de metaloproteinases, que desintegram a MEC promovendo uma desorganização estrutural do tecido. Estas enzimas agem ainda induzindo a diferenciação de adipócitos, que irão preencher o espaço desocupado pelo parênquima mamário (MACIAS; HINCK, 2012).

1.2 O USO E A AÇÃO DOS ESTRÓGENOS

Os estrógenos, hormônios sexuais femininos, compreendem uma classe de compostos esteroides caracterizados pela presença de 18 carbonos (Figura 3). Os principais estrógenos classificados como naturais são o 17- β estradiol, estriol e estrona; já o principal estrógeno classificados como sintético é o 17- α etinilestradiol (REIS FILHO; ARAÚJO; VIEIRA, 2006).

Figura 3. Estruturas químicas e principais vias metabólicas para síntese de alguns estrógenos. Adaptado de (THOMAS; POTTER, 2013).



O tratamento com estrógeno sintético tem sido recomendado para reposição hormonal em mulheres com sintomas de menopausa que incluem, como por exemplo, ondas de calor e atrofia vulvo-vaginal, como resultado de alterações vasomotoras (SOCIETY, 2010). Entretanto, pacientes submetidas a estes tratamentos hormonais têm um maior risco de desenvolver tromboembolismo, sendo estes muitas vezes contraindicados de acordo com o histórico médico (CANONICO

et al., 2010). Além disso, já é reconhecido o risco de desenvolvimento de câncer de mama em mulheres que fazem reposição hormonal após a menopausa (PIKE et al., 1993). Outra forma de exposição a estrógenos é por meio de pílulas anticoncepcionais, nas quais frequentemente utiliza-se o hormônio sintético etinilestradiol (DE LEO et al., 2016) ou valerato de estradiol (HOY; SCOTT, 2009), que comumente são associados a progestágenos.

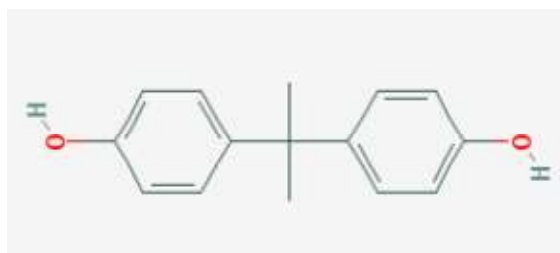
Além dos riscos oferecidos à saúde da paciente outra preocupação com relação ao uso farmacológico de estrógenos é o possível impacto ambiental. A contaminação do ambiente aquático ocorre, já que os metabólitos destes hormônios são excretados pela urina ou pelas fezes, sendo lançados como efluentes (ERICKSON, 2002; REIS FILHO; ARAÚJO; VIEIRA, 2006). De acordo com Johnson e Williams (2004) grande parte dos estrógenos administrados na população torna-se disponibilizada no ecossistema (JOHNSON; WILLIAMS, 2004). O mais preocupante é que apesar dos metabólitos representarem um menor risco com relação à contaminação ambiental, há a possibilidade de bactérias livres no ambiente, por exemplo, *Escherichia coli*, sintetizarem enzimas capazes de hidrolisar esteroides, transformando metabólitos inativos em moléculas ativas (TYLER; ROUTLEDGE, 1998).

Apesar dos preocupantes riscos sobre o uso de moléculas estrogênicas acima citados, a aplicação do 17- β estradiol durante os experimentos realizados para este trabalho teve o intuito de comparar a ação da fórmula mais adequada de ligação com o receptor de estrógeno e da ação do DE BPA, possibilitando verificar possíveis diferenças nas expressões de receptores hormonais bem como nas alterações morfológicas causadas por ambos após o tratamento perinatal.

1.3 O DISRUPTOR ENDÓCRINO BISFENOL A

Bisfenol A é o nome comumente utilizado para 2,2(4,4-dihidroxidifenil) propano, de acordo com a União Internacional de Química Pura e Aplicada. É um composto orgânico cujo peso molecular é 228,29 g/mol, constituído por dois anéis fenólicos ligados por uma ponte de metil acrescida de dois grupos metil (Figura 4) (MICHAŁOWICZ, 2014). Foi primeiramente desenvolvido como um estrógeno sintético já no final do século XIX mas seus efeitos no sistema reprodutor de ratas foi descrito somente nos anos 1930 (DODDS; LAWSON, 1936).

Figura 4. Estrutura química do BPA. (Pubchem Database 2018)



Sabe-se que este composto é bastante utilizado na indústria química durante a fabricação de resinas, plásticos policarbonatos, selantes e papéis térmicos (usados em faixas, rótulos, notas e recibos) (HOEKSTRA; SIMONEAU, 2013; LIAO; LIU; KANNAN, 2012). Desta forma, a população está em constante contato com BPA através do consumo de alimentos armazenados em garrafas plásticas e outros tipos de embalagens. Seu uso foi estabelecido devido à sua capacidade de inibir o fim da polimerização de cloreto de polivinil (PVC) bem como suas propriedades antioxidantes, além de ser um precursor para a síntese de componentes anti-chama (GEENS; GOEYENS; COVACI, 2011), proporcionando alta resistência à eletricidade e ao calor. Já as resinas epóxi são acrescidas de BPA para que sua dureza, resistência química e forte capacidade de adesão sejam melhoradas (www.factsaboutbpa.org, 2018).

Sua presença no ambiente ocorre exclusivamente devido à sua liberação dos componentes acima citados, sendo seu processo de produção industrial e degradação a principal fonte de contaminação de BPA para o ambiente (MERCEA, 2009). No caso de embalagens de PVC, baixo pH, alta temperatura e uso por muito tempo (3 a 7 anos) são fatores que aumentam a liberação de BPA tanto para o ambiente quanto para o conteúdo aquoso da embalagem. No caso deste conteúdo possuir algum teor alcoólico, a liberação de BPA é aumentada; já a presença de componentes oleosos reduz a eficiência de sua liberação (MERCEA, 2009). Se por um lado, a reação de hidrólise dos polímeros, responsável pela liberação de BPA, aumenta com altas temperaturas e tempo de uso, por outro ela é reduzida com o uso de detergentes durante as lavagens (MAIA et al., 2009). Devido à sua liberação também ocorrer no ar e na água há relatos da presença de BPA também na atmosfera, águas de efluentes e poeira, provando que a exposição pode se dar ainda pela via respiratória (MICHAŁOWICZ, 2014).

BPA é classificado como um DE, ou seja, é um composto que mimetiza total ou parcialmente a ação de hormônios endógenos potencialmente provocando uma hiperestimulação (NIH, 2018). É também classificado como um xenoestrógeno, uma vez que possui ações similares às daquelas dos estrógenos endógenos. Sua atividade estrogênica foi acidentalmente descoberta, quando produção de “estrógeno” foi identificada em cultura de levedura (FELDMAN et al., 1984). Após purificação da substância por HPLC a mesma foi identificada como BPA e descobriu-se que foi liberada dos frascos plásticos após autoclavagem (KRISHNAN et al., 1993).

Apesar de sua atividade estrogênica ser reduzida em termos de ligação ao ER- α e ER- β em comparação ao estradiol endógeno (1000 a 10000 vezes menor), BPA ocasiona diversas alterações nas funções celular e tecidual já que as moléculas podem ligar-se a receptores que não estão presentes no núcleo (WETHERILL et al., 2007). Além disso, sabe-se que seus metabólitos podem ser mais potentes em termos de atividade estrogênica do que 17- β estradiol (ALONSO-MAGDALENA et al., 2012). Foi sugerido também que o mecanismo de ação do BPA baseia-se em sua interação com receptores AHR (*aryl hydrocarbon receptor*), um fator de transcrição conhecido como um sensor de xenobióticos bem como um regulador do citocromo P450, enzima responsável pelo metabolismo destes compostos (ZIV-GAL et al., 2013).

Devido à sua estrutura química, BPA consegue ligar-se a ambos os receptores de estrógeno. Por serem fatores de transcrição, após o acoplamento de seu ligante, estes receptores mudam sua conformação e migram para o interior do núcleo com a função de regular a ação de genes alvo daquele hormônio (ASCENZI; BOCEDI; MARINO, 2006). Entretanto, a conformação do receptor pode mudar de acordo com o ligante (ASCENZI; BOCEDI; MARINO, 2006), de modo que a ligação do BPA, bem como outros xenoestrógenos, com ER constitui uma ligação inadequada, se comparada à conformação apresentada quando há ligação com estrógeno (ACCONCIA; PALLOTTINI; MARINO, 2015). Uma adequada disposição do domínio de ligação ao ligante é necessária para que ocorra uma conformação transcricional competente; conseqüentemente, variações ocorrem, em nível de expressão gênica (ACCONCIA; PALLOTTINI; MARINO, 2015; HONG et al., 2006).

A via de ligação ao receptor nuclear, contudo, confere ao BPA uma ação estrogênica relativamente fraca. Em termos de ligação a ER não localizados no núcleo, sabe-se que por meio das vias ERK/MAPK e PI3K/AKT – para ER α –, BPA

exerce sua função estrogênica mais significativa (MARINO et al., 2012), por meio de um mecanismo não genômico. Outro provável mecanismo não nuclear é a indução de liberação de Ca^{++} intracelular, modificando motilidade celular, processos de sinalização e exocitose (WOZNIAK; BULAYEVA; WATSON, 2005).

1.4 ALTERAÇÕES PATOLÓGICAS CAUSADAS POR BPA NA GLÂNDULA MAMÁRIA

Como já mencionado, o mecanismo de ação do BPA baseia-se em ligação a receptores celulares hormonais, de modo a bloquear ou modificar a ação dos mesmos. As consequências fisiológicas deste mecanismo variam de acordo com o órgão/sistema envolvido. De modo geral os efeitos pró-proliferação do ER- α e anti-proliferação do ER- β são perturbados com a ligação ao BPA. A ação agonista de ER- α e antagonista de ER- β promove exclusivamente um aumento na proliferação celular, impedindo o balanço entre as diferentes ações de ambos os receptores (ACCONCIA; PALLOTTINI; MARINO, 2015). Sendo assim, a principal alteração patológica causada em diversos órgãos por este composto é a hiperplasia (DURANDO et al., 2006). Mesmo quando a exposição ao BPA se dá no período perinatal, efeitos em longo prazo – durante a vida adulta – se fazem presentes, de modo que alguns autores atribuem estes achados ao aumento de estradiol sérico no organismo após a puberdade (DURANDO et al., 2006; MUÑOZ-DE-TORO et al., 2005).

Alterações em peso corporal, níveis hormonais séricos e distúrbios no padrão de ciclo estral são outros exemplos de alterações ocasionadas por BPA (RUBIN et al., 2001). As principais alterações morfológicas, genéticas, epigenéticas e de nível sérico hormonal ocasionadas por exposição ao BPA serão discutidas em detalhes a seguir.

1.4.1 Alterações genéticas e epigenéticas

Para a maioria das alterações causadas por aumento da exposição ao BPA há evidências de que causas epigenéticas estejam envolvidas. Alterações epigenéticas são aquelas que envolvem mudanças hereditárias e estáveis na expressão gênica sem alterar sua sequência de DNA (BERGER et al., 2009). Estas podem envolver mudanças no padrão de expressão e/ou silenciamento de genes e são ocasionadas

principalmente por meio de metilação – adição de um grupo metil ao nucleotídeo citosina presente em uma região citosina-fosfato-guanina (CPG) (MILEVA et al., 2014) – e mudanças em histonas (SENYILDIZ et al., 2017). Estes fatores de metilação podem ser transmitidos via material genético por várias gerações e podem, ainda, ser influenciados por fatores ambientais (CHONG; WHITELAW, 2004). O período de desenvolvimento perinatal é crítico no que se refere a estas influências, de modo que alterações epigenéticas ocorridas nos pais podem ser transmitidas aos filhos (MILEVA et al., 2014).

De acordo com Skinner et al (2010) a sequencia de eventos que culmina em efeitos patológicos inicia-se na ação epigenética, passando por alterações na leitura do DNA, de expressão de proteínas, na fisiologia do órgão até causar o distúrbio tecidual (SKINNER; MANIKKAM; GUERRERO-BOSAGNA, 2010). O efeito do BPA sobre a metilação de DNA em humanos (STRAKOVSKY; SCHANTZ, 2018) e em roedores (DOLINOY; HUANG; JIRTLE, 2007; YIN et al., 2016) já foi descrito. Alguns trabalhos descrevem a metilação causada por BPA em células do sistema nervoso. Yaoi et al (2008) relatam que a exposição materna ao BPA é capaz de alterar cerca de 0,3% das regiões CPG, causando hiper ou hipometilação no material genético do cérebro em desenvolvimento (YAOI et al., 2008). Estes autores reforçam ainda que as metilações causadas por BPA além de serem loco-específicas são ainda dependentes do estágio de desenvolvimento no qual o órgão se encontra.

Com relação aos genes possivelmente afetados, acredita-se que envolvam desregulação de, por exemplo, genes codificadores de receptores hormonais, alterando sua expressão ou sua atividade em longo prazo (CHIAM et al., 2009). Especificamente em células da GM alguns genes conhecidamente afetados pela ação *in vitro* do BPA estão envolvidos em reparo de DNA (BRCA1, BRCA2, BARD1, CtIP, RAD51 e BRCC3) e na regulação da apoptose (PDCD5 e BCL2L11) (FERNANDEZ et al., 2012).

1.4.2 Alterações morfológicas

A carcinogênese mamária é um processo complexo onde há alterações na interação epitélio/estroma (MAFFINI et al., 2005). A hipótese é de que a exposição a estrógenos ou disruptores hormonais durante o desenvolvimento perinatal influencie estas interações, ocasionando distúrbios na estrutura tecidual e na regulação proliferativa (DURANDO et al., 2006).

As alterações pré-neoplásicas baseiam-se no aumento do índice proliferativo associado à redução do índice apoptótico (DURANDO et al., 2006), culminando em hiperplasias ductais (MURRAY et al., 2007). Estes dois estudos avaliaram os efeitos da exposição perinatal ao BPA, levando à conclusão de que a exposição neste período é crítica e aumenta a sensibilidade da glândula ao hormônio endógeno, o que pode levar à malignidade em idades mais avançadas (MUÑOZ-DE-TORO et al., 2005).

Alterações a nível estromal também foram descritas. O crescimento desregulado do estroma por meio do aumento de proliferação de fibroblastos condiz com sua ação de apoio estrutural e hormonal quando a carcinogênese se inicia (DEB et al., 2004). Variações na deposição de fibras colágenas podem influenciar no crescimento celular e facilitar a metástase (AKIRI et al., 2003), sendo a MEC conhecida um importante fator regulador do câncer de mama (INSUA-RODRÍGUEZ; OSKARSSON, 2016). Mudanças nos locais de deposição e na densidade do colágeno na GM também foram relatadas em camundongos (VANDENBERG et al., 2007b).

O tecido adiposo que compõe a GM também pode apresentar alterações causadas pelo tratamento com BPA. Animais expostos apresentaram gordura significativamente menos densa do que os animais do grupo controle devido à redução na densidade celular e aumento no índice apoptótico, sugerindo que os achados são resultados da acelerada maturação dos adipócitos (VANDENBERG et al., 2007b).

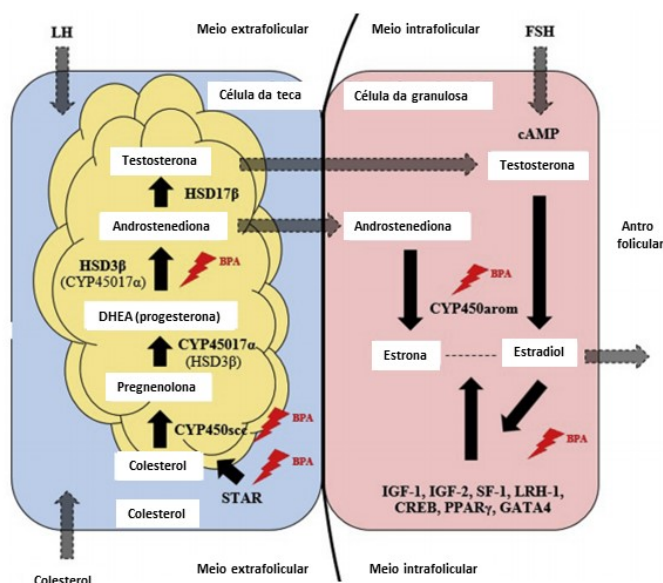
1.4.3 Alterações hormonais séricas

Há evidências de que BPA pode influenciar na produção de estrógenos pelas células da granulosa de folículos ovarianos, como demonstrado na Figura 5 (BLOOM; MOK-LIN; FUJIMOTO, 2016). Em estudos *in vitro*, Peretz et al (2010) relatam que BPA pode reduzir a esteroidogênese alterando suas vias sintéticas em células da teca e células da granulosa bem como limitando o crescimento folicular (PERETZ et al., 2010). Entretanto, o mecanismo envolvido nesta ação do BPA ainda é pouco esclarecido.

Estudos *in vivo* mostraram que os níveis de estradiol e de hormônio luteinizante (LH) foram maiores em ratos filhotes expostos durante gestação e lactação (GÁMEZ et al., 2015). Já em ratas avaliadas durante a idade adulta, a

exposição perinatal a BPA reduziu os níveis séricos de LH (RUBIN et al., 2001). Progesterona, entretanto, mostrou altos índices séricos aos 3 meses de idade em ratas também expostas durante seu período perinatal (SANTAMARÍA et al., 2016). Em animais pré-púberes o tratamento não resultou em alterações dos níveis séricos de estradiol, mas reduziu os níveis de progesterona (LI et al., 2014). De modo semelhante, a dose de 50 µg/kg/dia, considerada segura pela *Food and Drug Administration* (FDA), não promoveu diferença significativa entre os níveis séricos de estradiol, hormônio folículo estimulante (FSH) e LH de fêmeas de camundongos tratadas durante seus 3 primeiros ciclos estrais e aqueles dos animais dos grupos controle (MOORE-AMBRIZ et al., 2015).

Figura 5. Esquema mostrando as prováveis influências do BPA durante a esteroidogênese ovariana. A alteração na função enzimática ocorre devido à ação sobre proteínas esteroidogênicas da célula da teca – proteína reguladora de esteroidogênese aguda (STAR), proteína citocromo P450_{scc} (CYP450_{scc}), 3β hidroxisteróide desidrogenase (HSD3β) – e da célula da granulosa – aromatase (CYP450_{arom}) – bem como em alguns fatores – fator de crescimento semelhante a insulina I (IGF-1) e II (IGF-2), fator esteroidogênico 1 (SF-1), receptor homólogo do fígado (LRH-1), proteína de ligação ao elemento resposta ao AMPc (CREB), receptor ativo da proliferação de peroxissomos γ (PPARγ) e GATA4. Adaptado de Bloom et al 2016 (BLOOM; MOK-LIN; FUJIMOTO, 2016).



Os dados descritos na literatura revelam que alterações dos níveis hormonais séricos dependem do momento em que o BPA foi administrado nos animais. Os resultados descritos por Santamaría et al (2016) sugerem que BPA age nos primeiros estágios de desenvolvimento ovariano com consequências funcionais na

síntese hormonal da idade adulta (SANTAMARÍA et al., 2016). Um dos efeitos da exposição perinatal de BPA no eixo hipotálamo-hipófise-gônada é o aumento na expressão de mRNA de receptores de FSH.

2 JUSTIFICATIVA

Grande parte dos estudos realizados até hoje dizem respeito à morfofisiologia da GM de roedores de outras espécies, havendo limitada disponibilidade de informação sobre a GM dos gerbilos da Mongólia. Estes animais têm sido bastante utilizados em experimentos científicos. Isto porque, segundo Cheal (1986), esta espécie possui uma média de vida útil de aproximadamente 25,7 meses nos machos e 32,4 meses nas fêmeas, sendo relativamente curta (CHEAL, 1986). Além disso, apresentam um custo de manutenção acessível, são pequenos, relativamente fáceis de manipular e resistentes. Algumas áreas de estudo da Biologia, como genética (DE LA FUENTE et al., 2014), parasitologia (WEI et al., 2014), biologia celular e morfologia (WU et al., 2013) bem como oncologia (SALYARDS et al., 2013) também têm como modelo experimental já estabelecido o gerbilo. Em especial, em nosso laboratório, os mesmos servem como modelo experimental para estudos da morfologia, fisiologia e patologia prostática (CAMPOS et al., 2008), já que esta espécie conhecidamente apresenta altas taxas de desenvolvimento tumoral espontâneo (SALYARDS et al., 2013). Frente a esta característica, os gerbilos da Mongólia oferecem um ambiente ideal para estudos relacionados à carcinogênese, já que mimetizam, naturalmente, um ambiente propício ao desenvolvimento de tumores.

Os efeitos de DE em longo prazo são pouco descritos na literatura. Em especial, os resultados de sua exposição durante os primeiros estágios de desenvolvimento são pouco conhecidos. Apesar disso, alguns trabalhos disponíveis descrevem, por meio de análises diversas, que este período de desenvolvimento é crucial em termos de malignidade após a idade adulta.

Sendo os gerbilos animais que apresentam um organismo propício para o desenvolvimento de neoplasias espontâneas, acredita-se que isso possa mimetizar o ambiente carcinogênico a que os organismos estão sujeitos na idade adulta, como por exemplo consumo de álcool, cigarros e alimentos industrializados.

3 HIPÓTESE

A hipótese levantada para este trabalho é que o composto comumente utilizado na manufatura de produtos plásticos e resinas, BPA atue como um fator agravante para a ocorrência de desordens histopatológicas e moleculares mamárias em fêmeas adultas quando da prévia exposição perinatal do indivíduo.

4 OBJETIVOS

Este trabalho teve como objetivo avaliar os efeitos deletérios do BPA, em alta e baixa dose, bem como do 17- β estradiol na GM de fêmeas adultas de gerbilo da Mongólia expostas a estes compostos durante o período perinatal. Para uma melhor análise das alterações morfológicas objetivou-se também realizar uma descrição das características histológicas da GM nesta espécie em diferentes fases de seu desenvolvimento.

Desta forma, os objetivos específicos foram divididos em três diferentes etapas, a fim de avaliar os danos causados à GM a nível morfológico e molecular.

- Descrever e caracterizar morfológicamente a estrutura glandular mamária normal de fêmeas de gerbilos da Mongólia em diferentes estágios de desenvolvimento pós-natal em termos de composição tecidual, morfometria, expressão de receptores hormonais e índices proliferativos. GMs foram obtidas durante o estro, gestação, lactação e involução.

- Descrever as alterações morfológicas encontradas na GM de fêmeas adultas expostas a duas doses de BPA durante o período perinatal, levando em consideração a presença de alterações histopatológicas como hiperplasia, presença de células inflamatórias, destacamento de células epiteliais no lúmen, deposição de colágeno ao redor das estruturas epiteliais, análise da camada de células mioepiteliais, índice de proliferação celular e de expressão da proteína EZH2, relacionada à presença de alterações epigenéticas.

- Descrever as variações na expressão de receptores hormonais (ER- α , ER- β , PR, PRLR) por imunoistoquímica e western blotting além de quantificar os níveis séricos de estradiol e progesterona, com o intuito de verificar se há efeitos do BPA administrado no período perinatal sobre a ação hormonal nestas fêmeas.

5 RESULTADOS

Os resultados deste trabalho são apresentados em forma de artigos científicos de modo que cada um dos objetivos sugeridos é apresentado em um capítulo diferente.

O capítulo um apresenta os resultados já publicados na revista *Acta Histochemica* (ISSN: 0065-1281, 119 (3), 273-283, 2017, doi: 10.1016/j.acthis.2017.02.003), referentes à caracterização morfológica da GM de fêmeas de gerbilos da Mongólia em diferentes estágios de desenvolvimento.

Os capítulos dois e três apresentam os resultados obtidos no experimento também realizado com gerbilos da Mongólia em que foi analisado o efeito, em longo prazo, da exposição a diferentes doses de BPA, 17 β estradiol e veículo de administração dos compostos (óleo de milho) durante os períodos de embriogênese e lactação nas fêmeas adultas, aos seis meses de idade. O capítulo dois descreve as alterações morfológicas bem como expressão de proteínas relacionadas ao índice proliferativo e risco de desenvolvimento de câncer mamário. Este manuscrito foi submetido à revista *Experimental and Molecular Pathology* (ISSN: 0014-4800). Já o capítulo três discorre sobre os índices de expressão de receptores hormonais bem como as concentrações séricas de hormônios esteroides nos animais avaliados e foi submetido à revista *Ecotoxicology and Environmental Safety* (ISSN: 0147-6513).

O artigo produzido durante o período de estágio no exterior é apresentado ao final da tese, como Anexo. Este manuscrito foi submetido para a revista *Journal of Assisted Reproduction and Genetics* (ISSN: 1573-7330).

5.1 Capítulo 1



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Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution

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Abstract

The morphological description of normal tissues is fundamental for making comparisons and in order to identify injuries and lesions. The aim of this work was to describe the morphological characteristics of the female Mongolian gerbil's (*Meriones unguiculatus*) normal mammary gland, the average expression of the hormone receptors, and the average proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution. Dams were euthanized on the 14th and 21st gestational days, 7 and 14 days after birth, and 3 and 5 days after weaning. The dams' mammary tissues were processed and were submitted to haematoxylin and eosin staining, Periodic Acid Schiff (PAS) staining, and Gomori's Reticulin staining. Additionally, immunohistochemistry was performed for the characterization of the myoepithelial cells with α -actin, the proliferation rates with proliferating cell nuclear antigen (PCNA), the estrogen hormonal receptors (ESR1 and ESR2), and progesterone receptor (PR) quantifications. It was observed that the abundant adipose tissues were replaced by glandular epithelia and there was an increase in the epithelial cell's height (from 5.97 to 32.4 μm in gestation and from 20.64 to 25.4 μm in lactation) and the acini diameters (from 24.88 to 69.92 μm in gestation and from 139.69 to 118.59 μm in lactation) with the progression of the gestation and the lactation. The PAS staining intensity varied throughout the glands and between the stages that were evaluated. The extracellular matrix showed different phenotypes too, with more of a presence of the Type I collagen during the early gestation and

involution and with more reticular fibers (Type III collagen) during the late gestation period and lactation. The myoepithelial layers showed alterations in their distribution with thick patterns as verified by the α -actin labeling. The PCNA showed higher rates of the marked cells during the gestation (40.25 and 60.28%) and the lactation (64.08 and 65.08%). The hormone receptor quantifications showed a high variation in the rates: the PR showed lower averages with the progression of the gestation (from 42.3 to 8.54%), the lactation (from 59.83 to 23.18%) and the involution (from 39.98 to 12.72). ESR1 showed higher averages during pregnancy (from 58.06 to 30.02%) and ESR2 during the gestation (from 25.7 to 12.94%), which was reduced through the involution (from 50.97 to 30.18%). The Mongolian gerbils showed similar morphological characteristics when they were compared to mice and rats. However, the higher proliferation rates with a smaller involution period compared to other murine characterized this species as being adequate for mammary pathologies studies.

Keywords: gerbil, mammary epithelium, morphology, cell proliferation, immunohistochemistry, hormonal receptor

1. Introduction

The mammary gland, which distinguishes mammals from all other animals, produces and secretes milk in order to nourish offspring. It is classified as apocrine exocrine tubuloalveolar (Inman et al., 2015; Oftedal, 2002). Although there are structural differences between species, several researches use the mouse and rat mammary gland as a model to infer about developmental mechanisms (Macias and Hinck, 2012; Howlin et al., 2006). In general, the primary mammary gland structure originates from the ectoderm (epithelium) and the mesoderm (stroma). The interaction between these tissues promotes the formation of the mammary placodes which develop towards the anterior portion of the body (Balinsky, 1950). Generally after birth, the gland remains quiescent until the beginning of the cyclic reproductive life, in response to hormonal cues. Thus, the mammary gland provides a unique model for biologists to study development and organ specificity (Inman et al., 2015). In rats, the parenchymal compartment of the mammary gland is composed of different epithelial structures with distinct morphological and functional activities, comprising the luminal epithelium of ducts, ductules, terminal end buds, alveolar buds, alveoli and the underlying myoepithelial layer (Liška et al., 2016; Masso-Welch et al., 2000). As in most glandular tissues, the adult mammary gland comprises multiple cell types, including epithelial, adipose, fibroblasts, immune, lymphatic and vascular cells that work together to sculpt and maintain a functional organ. These different cell types have been demonstrated to be of importance at specific stages of mammary gland development (Inman et al., 2015). The increase of estradiol secretion during puberty promotes the storage of dense connective tissues and adipocytes among the lobules (Sinowatz, 2012). Between the luminal epithelium and basement membrane the contractible myoepithelium is present, promoting the milk excretion under oxytocin stimulation during pregnancy (Masso-Welch et al., 2000). The gland undergoes deep modifications with its structure during the reproductive cycles due to the hypothalamic-hypophysis-gonadal axis activity. In nonpregnant female, the development of the mammary gland is rigorously controlled by the ovary. In general, estrogens are responsible for mammary ducts growth and progesterone is necessary for lobuloalveolar development in the mouse (Russo and Russo, 1996). Nevertheless, the functional and structural development occurs in the dam exclusively during gestation and lactation, followed by involution (Sinowatz, 2012).

During these different stages, the cells of the mammary gland proliferate, differentiate and go through apoptosis in response to stimuli, giving rise to significant remodeling of the glandular tissue architecture (Inman et al., 2015).

In the dam, early in gestation, the combined influences of ovarian estrogen, progesterone, and inhibin, with the production of rat chorionic gonadotropin contribute to stimulate the gland to undergo active cell proliferation (Ying, 1988). In the gestational and the lactational periods, the morphological alterations in the dam include an alveolar expansion of the secretory epithelium regions towards the fat pad, culminating in a considerable remodeling of adipose tissue (Collins and Schnitt, 2012). The end of gestation is the lobuloalveolar phase of the mammary gland's development (Richert et al., 2000). In C57BL/6 mice during lactation, the pre-secretory cells go through an intense activity, releasing their components, together with apical region pieces of the cytoplasm to the lumen. In this strain, after the end of lactation, the absence of a mechanical suckling stimulation promotes the mammary involution: apoptosis, epithelial cell detachment, and an alteration of the acini's shape are characteristics of the first stage of mammary involution (Inman et al., 2015; Monks et al., 2008). In rats, together with the secretory alveolar collapse, there is removal of active cells and secretions by macrophages (Helminen et al., 1968). In human mammary involution the extracellular matrix remodeling that is associated with a new wave of apoptosis then occurs, promoting the substitution of the epithelium by the adipose tissues (Macias and Hinck, 2012). This process of expansion and regression can occur across multiple gestations during the reproductive phase, demonstrating that the mammary epithelial cells have considerable regenerative abilities (Arendt and Kuperwasser, 2015).

In female mice offspring, on day 14 of gestation, the epithelium of the mammary gland responds to estrogen, thanks to the presence of estrogen receptors (ESR) (Sampayo et al., 2013). In the adult mammary gland, however, only a small population of epithelial cells expresses ESRs and progesterone receptors (PRs). It has been stated in several species and, although not all of their functions are clear, it is known that ESR1 is the most important receptor during the mammary ductal morphogenesis. PRs are only necessary during alveologenesi and throughout the gestation period (Macias and Hinck, 2012). Despite ESR1 is related to cellular proliferation, it is known that it exerts a paracrine effect, since the ESR1 positive cells have shown that they are not the same as those that are positive for

bromodeoxyuridine in Balb/c mice (Zeps et al., 1998). A paracrine mechanism is also shown by the PR-positive cells in mammary gland of ROSA26 and RAG1 mice (Briskin et al., 1998). Moreover, estrogen receptor gene ESR2 knockout mice have shown no deficiencies in the mammary structure or with lactogenesis (Mehta et al., 2014; Krege et al., 1998). This suggests a greater presence of ESR1 and PR during the development stages (regulating ductal outgrowth and morphogenesis) while ESR2 is associated with the non-proliferative phases of the gland (Macias and Hinck, 2012). The ESR1-knockout mouse is infertile and it presents a rudimentary development of the mammary ductal system, because it lacks the terminal end buds. It seems that the growth of ducts depends on the presence of ESR1 in the stroma, and that the availability of epithelial cells expressing ESR2 is insufficient to evoke a mammary proliferation induced by estrogen (Hamilton et al., 2014; Musumeci et al., 2015). ESRs and PRs are also present in mesenchymal cells surrounding the mammary gland (Bigsby et al., 2004).

In both rats and mice, the mammary glands are aligned ventrolaterally along the mammary or milk lines from cervical to the inguinal regions. Female mice have five pairs of mammary glands: one cervical, two thoracic, and two abdominal-inguinal pairs. The female rat has six pairs, the thoracic, abdominal, and inguinal glands that vary in their degree of development in the nulliparous rats, with the inguinal being the most differentiated, and the cervical glands being the least differentiated. The anatomic location and distribution of these paired organs is similar between these two species (Russo and Russo, 1996).

Female Mongolian gerbil (*Meriones unguiculatus*), a polyestrous species, have an estrous cycle of 4–6 days (Nishino and Totsukawa, 1996) and reaches puberty at 9–12 weeks. Gestation lasts 24–26 days and weaning happens 3 weeks after birth (Marston and Chang, 1965). They show four pairs of mammary gland: two thoracic and two inguinal (Fig. 1). Each of them show only one galactophore duct (Yu and Anderson, 1975).

A morpho-physiological description of the mammary gland has been made when related to several species (Bellatine et al., 2010; Chandra et al., 2010; Saji et al., 2000; Zeps et al., 1998). However, a descriptive study about the morphological variations of a Mongolian gerbil's mammary gland is not available in the literature. These experimental models have been increasingly used due to their relatively high development rates of spontaneous tumors (Salyards et al., 2013; Campos et al.,

2008), what mimics the natural tumor environment and enables the development of research in this particular area. Thus, the aim of this study was to describe the structural characteristics of the female Mongolian gerbil's mammary gland, the average patterns of the hormone receptor expression, and the average proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution as a contribution to understanding the various aspects that involve the development and functionality of these glands.

2. Material and Methods

2.1 Animals and experimental design

The animals were kept under standard temperature and humidity conditions (25 °C, relative humidity 40–70%, light/dark cycle 12/12 h) with access to chow (Rats and Mice, Presence®), and water ad libitum. The procedures were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and was approved by the Ethics Committee on Animal Use (CEUA-IBILCE/UNESP 099/2014).

The study used 7 young adult (4 months old) female Mongolian gerbils (*Meriones unguiculatus*). One of them was a 4 month old female in estrous phase, whose mammary gland was used as resting control for comparison to other animals. The other six animals were housed with fertile males for the mating. All animals were euthanized at various stages of their reproductive life. The mating day was determined by the presence of sperm using a vaginal smear, indicating day 0 of pregnancy. One animal was euthanized at each of the following periods: the 14th and 21st days of pregnancy, 7 and 14 days after the birth of the puppies (lactation), and 3 and 5 days after the weaning (involution).

2.2 Histological analyses

Euthanasia was performed by a deepening anesthesia (xylazine 3 mg/kg and ketamin 10 mg/kg) followed by decapitation. After death, the abdominal area was shaved, enabling a visualization of the left caudal mammary glands, which were excised with the skin. The glandular tissue samples were fixed for 24 h in 4% paraformaldehyde that was diluted in a phosphate buffer solution. They were then processed for histology and cut with a microtome (4 µm thick longitudinal sections).

Two sections were analyzed per gland. They were stained with Haematoxylin and Eosin (HE) for a general morphological analysis. A Periodic Acid Schiff (PAS) staining was performed, in order to identify the carbohydrates in the secretion. Gomori's Reticulin staining was performed too for the identification and the characterization of the reticular fibers in the stroma. The sections were analyzed under a light microscope (Olympus BX60, Japan) and the images were digitized and analyzed by the Image Pro Plus software (Version 6.1 for Windows – Media Cybernetics, Silver Spring, MD, USA).

2.3 Morphometric analyses

One section per animal was scanned using a B61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5) from the same manufacturer and the total area of the section was measured. The ratio between the area occupied by the secretory epithelium and the area that was occupied by the fat cells and the stroma were calculated in μm^2 by the Image Pro-Plus software (Image ProPlus Media Cybernetics, MD, USA).

Representative fields scattered in the preparation (chosen randomly) of the HE stained sections were also analyzed in order to obtain the measurements of the acini diameters and the glandular secretory epithelium heights. It was analyzed two slides per animal. For the acini diameters, the mean and standard deviations of the values that were obtained for one mammary gland per animal were calculated from 60 measurements per gland/animal. To calculate the overall average of the epithelium height, 12 acini were evaluated and three cells of each were measured.

2.4 Immunohistochemistry

The sections that were obtained from each animal were submitted to immunohistochemical reactions with the primary antibodies for ESR1 (ER α , rabbit polyclonal, IgG, MC-20, sc-542, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ESR2 (ER β , rabbit polyclonal, IgG, H-150, sc-8974, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PR (rabbit polyclonal, IgG, C-19; sc-538, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) receptors. Also, Proliferating Cell Nuclear Antigen (PCNA) which is involved in cell proliferation and DNA repair antibody (mouse monoclonal, PC10, dilution 1: 100, sc-56, Santa Cruz

Biotechnology, Santa Cruz, CA, USA) was used. The antigen retrieval was performed by an immersion of the sections in 10 mM citrate buffer, pH 6.0, at 93 °C for 20 (for PCNA protocol) or 45 min (for hormone receptors protocols). A blockage of the nonspecific proteins was then carried out with a skimmed milk powder solution of 5% for 30 (for ESR1 and ESR2 protocols) to 60 min (for PCNA protocol), or with sniper background (Biocare Medical, Concord, CA, USA) for 15–20 min (for PR protocol). The sections were incubated overnight at 4 °C (for hormone receptors protocols) or for one hour at 36 °C (for PCNA protocol) with the primary antibodies and then incubated with the secondary antibodies for 1 h (Goat anti-rabbit IgG, ABC Staining Systems, Santa Cruz Biotechnology, CA, USA for hormone receptors protocols and goat anti-mouse IgG, sc-2039, dilution 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA for PCNA protocol). After that, an incubation with avidin/biotin (ABC Staining Systems, Santa Cruz Biotechnology, CA, USA) for hormone receptors protocols, or polymer (DAKO EnVision™, Dual Link System-HRP, Dako, CA, USA) for PCNA protocol, for 45 min at 22 °C, was performed. The sections were stained with a solution of 3–30 ' -diaminobenzidine tetrahydrochloride (DAB, Sigma), and counterstained with Harris Haematoxylin.

2.5 Immunofluorescence

Sections were also submitted to immunofluorescence for the detection of the myoepithelial cells by α -actin (mouse monoclonal, IgG2a, 1A4, sc-32251, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation with the primary antibody was performed overnight at 4°C, followed by an incubation with specific secondary fluorochrome-conjugated antibody (anti-mouse goat, IgG-FITC, sc-2010, dilution 1: 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for two hours at 22°C. DAPI (4,6-diamino-2-phenylindol, UltraCruz™ Mounting Medium: sc-24941 for fluorescence with DAPI, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for a visualization of the cell nuclei. The sections were examined with a fluorescence microscope (Zeiss Imager M2, Zeiss, Gottingen, Germany).

2.6 Cellular proliferation rates and the quantification of ESR1, ESR2 and the PR positive cells

Six representative microscopic fields (40× magnification) were analyzed per animal in order to quantify the relative frequency of the positive cells for ESR1, ESR2, PR and PCNA in the secretory epithelium of the mammary gland. The percentages were calculated by a division of the number of labeled nuclei by the total number of cells counted in each field.

3. Results

3.1 The adipose tissue was partially replaced by the glandular epithelial cells. The extracellular matrix fibers underwent variations in their organization and the secretion compositions varied through the lobes during their functional differentiation

The mammary glands at different stages of the reproduction showed considerable variations in their morphology (Figs. 2 and 3).

Compared to the resting gland, it was observed that on the 14th gestational day, the mammary gland had a higher frequency of mammary epithelium (Fig. 3A–C). At this stage, there were small quantities of an eosinophilic secretion in few of the acini, with the presence of lipid droplets in their composition. The acini comprise a simple cuboidal epithelium. The final portion of the ducts was characterized by a simple squamous epithelium. The PAS staining was less intense in some acini when compared to the secretion that was present in the ducts. From 14 days of gestation on, the highly PAS positive secretion was associated with lipid droplets, characterizing the beginning of the milk production. The adipose tissue, which constituted the most part of the glands, was classified as unilocular. It was partially replaced by the secretory epithelium and loose and cellularized connective tissues, reducing the adipocytes contribution to the gland's composition. Gomori's Reticulin stain revealed thick bands of collagen fibers (Type I) amid the stroma, delimiting the areas of the glandular lobules. The acini and the ducts were externally delimited by a thin layer of black reticular fibers (collagen Type III).

In the last gestational week (21 days), there was a larger area occupied by the secretory epithelium, and consequently, there were less unilocular adipose tissues (Fig. 3D–F). The morphologic profile of epithelial cells altered, so that the acini were smaller at this stage of the development. The cells showed an extended cytoplasm, comprising a higher epithelium. The presence of blood capillaries in the acini became more evident, with an easy identification of red blood cells in the acinar baseline

limits. The distribution of collagen Type I fibers at this stage was greatly reduced when compared to the previous phase and a thin layer of reticular fibers limited the alveoli. Nevertheless, the dense connective tissues retained their irregular characteristics as was seen at 14 days of gestation.

Considerable morphological changes occurred in the mammary gland during the perinatal period (Fig. 3G–M). A thin periglandular stromal layer filled the glands and a pronounced development of the secretory epithelium and the ductal structures was observed at 7 days postpartum – lactation. The adipose tissues were limited to small portions in the peripheral regions of the glands, with a considerable reduction in their size. The acini became considerably larger. The epithelial cell apical delimitations were not clear and this particular region of the cell consisted mostly of vesicles and lipid droplets. A small amount of loose and cellularized connective tissues delimiting the alveoli was richly vascularized. Cells with condensed chromatin corpuscles were identified in the lumen, corresponding to the detached cells that were released from the secretory epithelium. At this stage, the secretion stained by the PAS was less intense indicating a secretion with a lower carbohydrate concentration.

After the end of the lactational phase (Fig. 3N–T), the amount of detached epithelial cells in the lumen increased, becoming more frequent during the regression of the glands. Despite the absence of mechanical suction stimuli, three days of post-weaning acini remained morphologically similar to that which was shown during lactation, with stored secretion. Five days after the weaning, significant changes were observed. The acini showed a reduction in their size and frequency, the adipose tissues returned to occupy a considerable part of the glands and lumen showed remains of PAS positive secretion. In a similar manner to that which was observed during the resting phases, the Type I collagen fibers occupied much of the stroma. In contrast, the reticular fibers considerably reduced.

3.2 The acini diameters and the epithelium heights increased with the progression of pregnancy and reduced considerably during involution

The mean and the standard deviations of the secretory acini diameters and the epithelial heights are shown in Fig. 4.

At 21 days of gestation, the acini increased their diameter by about three times when compared to the 14 day of this period. The average values of the secretory epithelium heights showed an increase of 6 times over gestation. The measures

doubled after the onset of lactation and they remained relatively high until the lactational end. However, a slight decrease in the epithelium height averages, from the late pregnancy (32.4 μm) to the first week of lactation (20.64 μm), was observed. After the end of lactation, the parameters became similar to those that were observed at the beginning of lactation.

The values of the ratios between the areas occupied by the secretory glandular epithelium and the stroma are shown in Table 1 and are given in μm^2 . It was observed that there was a gradual replacement of the fat pad by the secreting epithelial tissues during pregnancy and lactation. After weaning, involution occurred in a fast period, reaching a ratio epithelium/adipocytes and stroma similar to that observed at 14 days of gestation five days after weaning.

3.3 Immunohistochemistry

3.3.1 The Myoepithelial Cell Layer Was More Remarkable During Pregnancy

The images of the α -actin labeled cells that were obtained by the fluorescence microscope are shown in Fig. 5. The immunofluorescence for α -actin revealed that the myoepithelial cells were arranged concentrically around the acini and ducts.

The images of the hormonal receptors and PCNA positive cells are shown in Fig. 6.

3.3.2 The PCNA expression increased in the epithelial cells at the end of pregnancy and during lactation

An increase in the epithelial cell proliferation rates was observed at 21 days of gestation (60.28%) and during lactation (64.08 and 65.08%) when compared to the 14 days of gestation (40.25%).

The PCNA labeling rates reduced in involution period (57.34 and 52.38%) compared to late gestation and lactation, but without reaching the levels that were presented during the early gestation (Fig. 6A).

3.3.3 The hormone receptor expressions varied at different stages of the mammary gland development

The hormone receptors positive epithelial cells were identified by immunohistochemistry as a brown nucleus in appearance and quantified (Fig. 6B–D).

The percentages of the PR positive cells showed large variations between the animals. During pregnancy, the values showed a sharp drop in PR positive cells from 14 days (42.3%) to 21 days (8.5%), then going up during lactation (59.8 and 23.18) and involution of the glands (39.9 and 12.72%).

The ESR1 positive cells showed increases and reductions, similar to the PR. There was a decrement from 14 days (58.0%) to 21 days during gestation (30.0%). Lactation showed a precipitous drop on day 7 (7.1%), followed by an increase on day 14 (31.9%), with declination during involution periods (26.6 and 9.5%). The ESR2 positive cell percentages showed lower values during pregnancy (25.7 and 12.9%), which increased during lactation (60.6 and 68, 2%) and involution (50.9 and 30.1%).

4. Discussion

Although the anatomical features and a little of the Mongolian gerbil's mammary gland histology have been previously described (Yu and Anderson, 1975), the present study adds a considerable morphophysiological description of this species. It was described the structural characteristics of the gerbil's mammary gland, the hormone receptor expression pattern, and the proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution.

There was a clear change in the glands that provided development of their secreting potential during the periods of pregnancy and lactation. During the early stages of pregnancy, implantation of the embryo in the uterine wall induced the maintenance of the corpus luteum in the ovary and increased the levels of progesterone. As a consequence, the gland now develops itself, from its rudimentary structures, to exert its function. Firstly, the ductal tree develops from the activation and the proliferation of the TEBs, where there was a differentiation of the secretory epithelium, thus developing the acinar structure. To this aim, the unilocular fat cells showed a reduction in their size, since they provided energy and substrates for the synthesis of the growth factors and the hormones necessary for the differentiation and lactation, apart from yielding space for the development of the ducts and the acini (Hovey and Aimo, 2010). As in several rat strains (Liška et al., 2016), the gerbil's mammary gland had a higher amount of fat in their non-secretory portions.

It was observed that the growth of the secretory portions during gestation was accompanied by extracellular matrix remodeling, as in mice strains (Silberstein, 2001). Also, the epithelial cells were always surrounded externally by a collagen

layer, which was thicker in the early pregnancy period and less developed in late gestation and early lactation. The epithelium of the ducts that were observed in gerbils varied from a cuboid monolayer, similar to that observed in dogs (Chandra et al., 2010) and pigs (Ji et al., 2006), to a simple squamous, when it was closer to the secretory acini. Also, the acini epithelium in the active gland of gerbils was classified as a simple cuboidal epithelium.

Once the alveoli were developed and the epithelium differentiated as secretory cells, the epithelial height, and consequently, the cell volume increased with gestation. The results that were observed during the analyses of the epithelial height measurements and the acini diameters led one to conclude that a higher epithelial height was observed during pregnancy, the same as in rats (Líska et al., 2016). A deposition of fat droplets and secretion vesicles in the cytoplasm of the secretory cells was responsible for the increase in its volume. During pregnancy, the ovarian progesterone production suppressed the milk secretion (Neville et al., 2002), which was activated in the perinatal period with the production of colostrum and milk. In late gestation, the accumulated content in the cytoplasm was transferred to the lumen and the myoepithelial cells underwent a multiplication process and reach their peak during the early stages of lactation, as has been described in Wistar rats (Jin et al., 2000). From now on, their morphology was changed to a thicker aspect that surrounded the largely developed acini during the late gestation and early lactation. During pregnancy, it was observed that the myoepithelial cell layers were continuous in the ducts and discontinuous in the acini, providing a greater contractility for the excretory portion of the gland, similar to that seen in Sprague-Dawley rats and canines (Sanchez-Céspedes et al., 2016; Masso-Welch et al., 2000).

The percentages of the PCNA positive cells increased during pregnancy. This marker has also been related to the DNA repair (Shivji et al., 1992), but the increase in its rates during pregnancy may be associated with cell proliferations. Although ESR1 and PR are related to proliferation, the quantitative analyses showed a reduction in these receptor rates during the periods of increased positivity for the PCNA. As described by Shigehira et al. (2000), the presence of estrogen receptors in epithelial cells is not a prerequisite for proliferation. Therefore, these results have corroborated the assumption of the paracrine proliferation effects as shown by the PR (Briskin et al., 1998) and the ESR1 (Zeps et al., 1998) positive cells in the mammary glands of mice. ESR2 knockout mice have shown that female fertility and the development of

mammary glands during pregnancy and lactation are not altered when in comparison to wild animals (Krege et al., 1998). Also, it is known that the ESR2 expression naturally exceeds the ESR1 expression in mammary glands and has an antiproliferative action, as in other tissues (Mehta et al., 2014). According to Cheng et al. (2004), ESR1 is directly associated with the cellular proliferation because it initiates the DNA synthesis, and thereafter, it ceases to be expressed in the nucleus. ESR2, in turn, serves to facilitate the ESR1's return to the nucleus and resumes the responsiveness to the hormone. This may explain the high rates of ESR2 positive cells in times of a low expression of ESR1.

During lactation, a drop in the serum progesterone that is associated with other endocrine changes leads to a milk secretion. For the milk production, the loss of cytoplasm in the epithelial cells is necessary, featuring a mixed apocrine secretion, containing lipids, lactose and proteins. This cell content loss causes a decrease in the average heights of the secretory epithelium. The difficulty in defining the limits of the apical secretory cells at this stage confirms this proposition. The different PAS staining shades that were observed throughout the mammary glands may be related to the changes in the molecular composition of secretion during gestation, lactation and involution. According to Grigor et al. (1986), carbohydrates concentration in mammary gland secretion may vary through the glands according to the suckling frequency, which may explain these differences in PAS staining. In C57BL/6J and SEG/Pas mice, the glycosylation of the proteins secreted by the mammary gland in the beginning of lactation is more intense than that observed in the end (Boumahrou et al., 2011). This is an indicative that the stage of lactation affects the composition of the secretion. In lactation, between adjacent secretory epithelial cells, permeable tight junctions are formed to prevent the leakage of milk components after parturition, concurrent with lactogenesis (Kobayashi et al., 2016). These junctions are formed during lactogenesis and are instrumental in establishing and maintaining milk synthesis and secretion. However its integrity is compromised during mammary involution (Stelwagen and Singh, 2014). The myoepithelial cells had a full development during lactation and their appearance at this stage was similar to that described for rats: increased cytoplasmic processes for a greater coverage of ducts during lactation (Jin et al., 2000), however, being discontinuous around the acini (Masso-Welch et al., 2000).

The increase in the PCNA rates throughout lactation indicated cell proliferation during this period, as has been described for mice (Traurig, 1967), with a peak reached in early lactation (Liška et al., 2016). In contrast, apoptosis was present in the gerbils and was identified by the presence of apoptotic bodies in the lumen, showing cell renewal.

During lactation, the ESR2 expression exceeded that of the ESR1 expression. This showed that at this stage of the mammary gland's development, although the proliferation rates were high, the return of the ESR1 receptors to the nucleus was probably occurring, as described by Cheng et al. (2004), also indicating a differentiation of the epithelial cells. As in cows (Schams et al., 2003) and sheep (Colitti and Parillo, 2013), the ESR1 receptors were also present in the cytoplasm. In sheep, the peak percentages of the PR positive cells also occurred in early lactation (Colitti and Parillo, 2013).

The mammary involution after weaning was associated with the epithelium compression, due to the milk accumulation in the lobes, together with ischemia and with an absence of mechanical stimuli suction, and even, the action of digestive enzymes present in the basal membrane (Masso-Welch et al., 2000). According to Macias and Hinck (2012), the rodent mammary involution is divided in two stages, which may be applicable for involution process observed in gerbils. The first stage is reversible and lactation can be resumed with the return of the suction stimuli. The second stage is irreversible, as the alveoli begin to collapse and the return of suction does not resume lactation. According to our results, the second phase of involution in the gerbil's mammary glands started between the third and fifth days of post-weaning, since the morphological changes were not showed on the third day, but were identified on the fifth day. The secondary involution process was more evident after 5 days of weaning and this has also been described in rats, with a greater increase in the proportion of fat when in relation to the secretory epithelium (Liška et al., 2016). According to Liška et al. (2016), each development stage in the rat's mammary gland has a specific composition of the extracellular matrix, which aids in the mammary differentiation, justifying the variation of the fiber types that were observed during the period that was assessed for the gerbils. The mammary regression procedure in the gerbils occurred faster than the development that was observed during pregnancy, since the epithelium predominance that was observed at regression day 5 was very close to that observed at gestational day 14. The values

for the acini diameter, the epithelium height, and the epithelium area, supported the conclusion that at post weaning day 5, the mammary involution was already in advanced stages, with similar aspects to those that were shown in early pregnancy. Watson (2006) stated that in mice, involution of the mammary epithelium was concluded six days after the 10-day post-partum forced weaning. In rats, the adipose tissue percentages at involution day 5, is statistically similar to that observed at the beginning of lactation (Leite et al., 2007). Thus, the mammary involution in the gerbils was quicker than in mice and rats, since the adipose tissue area percentages at post-weaning day 5 were very close to those shown at the beginning of gestation. For most species, the loss of cell-cell communication may initiate involution and apoptosis of mammary epithelial cells and is a localized intramammary event, occurring only in non-suckled glands (Phyn et al., 2016). The neighboring mammary epithelial cells themselves appear to be the primary cell type responsible for apoptotic cell clearance during involution. Although there is increasing evidence that phagocytosis by mammary epithelial cells has a crucial role in maintaining tissue homeostasis in the involuting murine mammary gland, little is known about how mammary epithelial cells become phagocytic during postpartum involution (Fornetti et al., 2016).

The hormone receptors showed a small increase in their expression during the mammary involution when compared to the lactational period. In bovine mammary glands, the expression of ESR1 and PR is similar to that observed in the present experiment, with a decrease during lactation and small increase during the mammary involution (Schams et al., 2003). These observations have also inferred about the possible role of estrogen signaling during the gland involution period. The mammary glands underwent a considerable morphological change, which was based on a reduction of the unilocular adipose tissues during pregnancy and that was associated with the development of the mammary secretion epithelium. The cell proliferation rates remained high until the end of lactation and the expression of the hormone receptors varied considerably between pregnancy and involution. At the end of the intense production season, the glands rapidly restored their standards, substantially reducing the amount of mammary ducts and alveoli, and presenting again, a large unilocular fat deposition. Considering the regenerative abilities of the mammary gland during the reproductive phases and its high propensity to develop neoplastic lesions, new experimental models may help the understanding of molecular processes that

happen in this organ. Thus, once the number of published work about Mongolian gerbils is scarce, this work helps the elucidation of its morphological characteristics. In conclusion, the Mongolian gerbil's mammary gland morphophysiology shows similarities to that of other rodents. However, a faster involution period and high proliferation rates have featured this animal as an excellent experimental model for mammary gland research. Our results have proved that Mongolian gerbils may be suitable for clarifying the molecular and morphological aspects of healthy and pathologic mammary gland.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.acthis.2017.02.003>.

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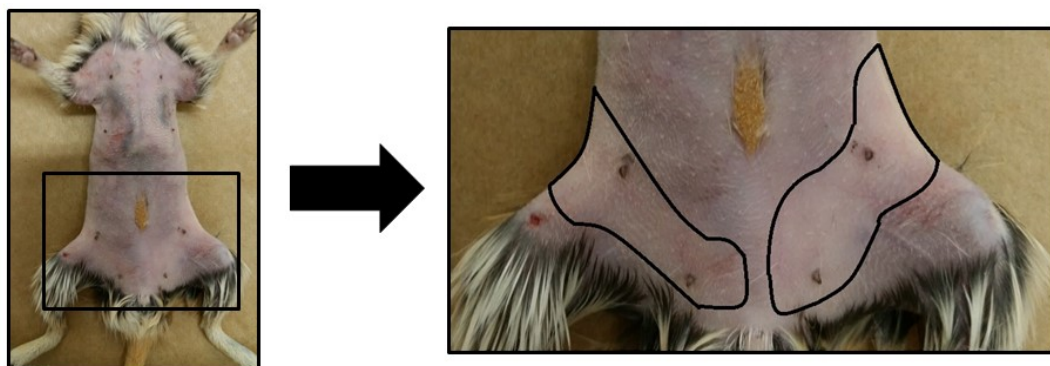


Fig 1. Schematic illustration of the inguinal mammary glands anatomic position of the female Mongolian gerbil.

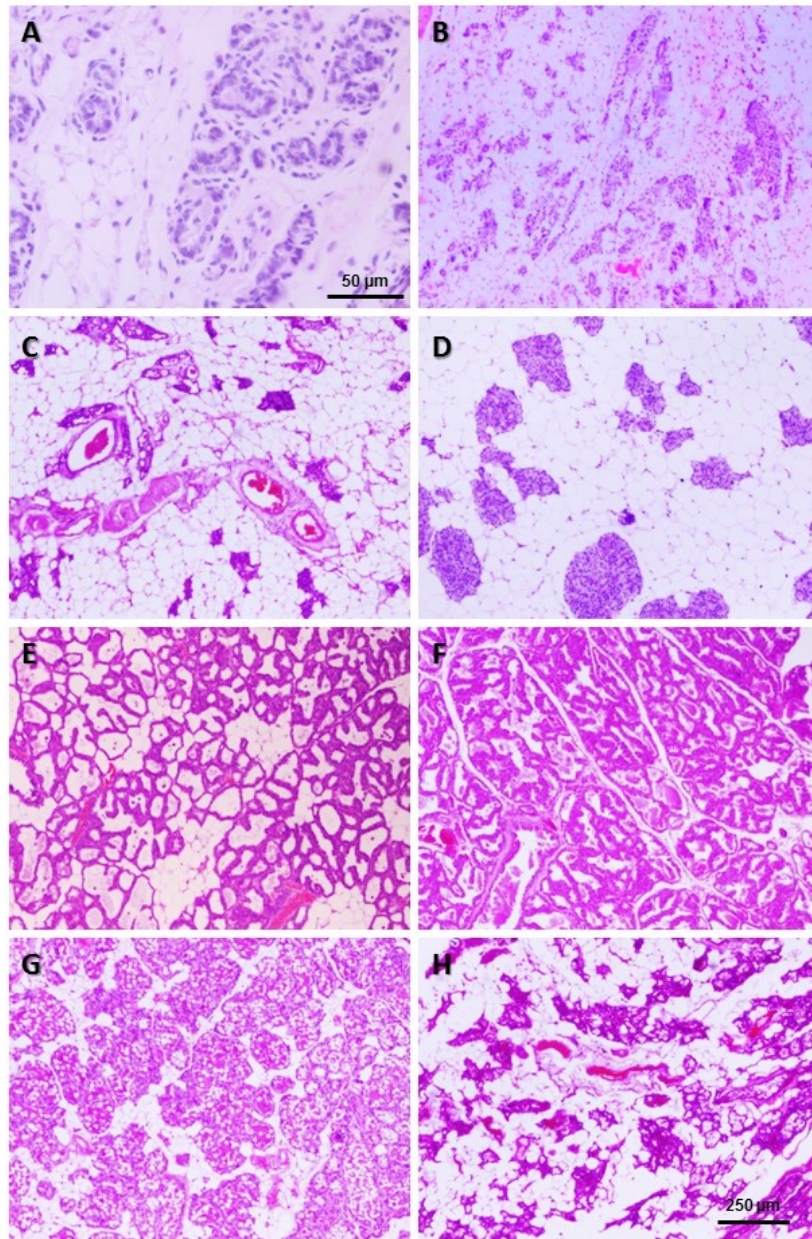


Fig. 2. Morphological aspects of female gerbil mammary gland in low magnifications. Resting gland from female at estrous (A and B), 14 days of gestation (C), 21 days of gestation (D), 7 days of lactation (E), 14 days of lactation (F), 3 days after weaning (G) and 5 days after weaning (H) stained with haematoxylin and eosin are shown. The main difference noticed between the stages is the frequency of epithelial structures amid the adipose tissue. Magnifications: A-G: 4x; H: 20x.

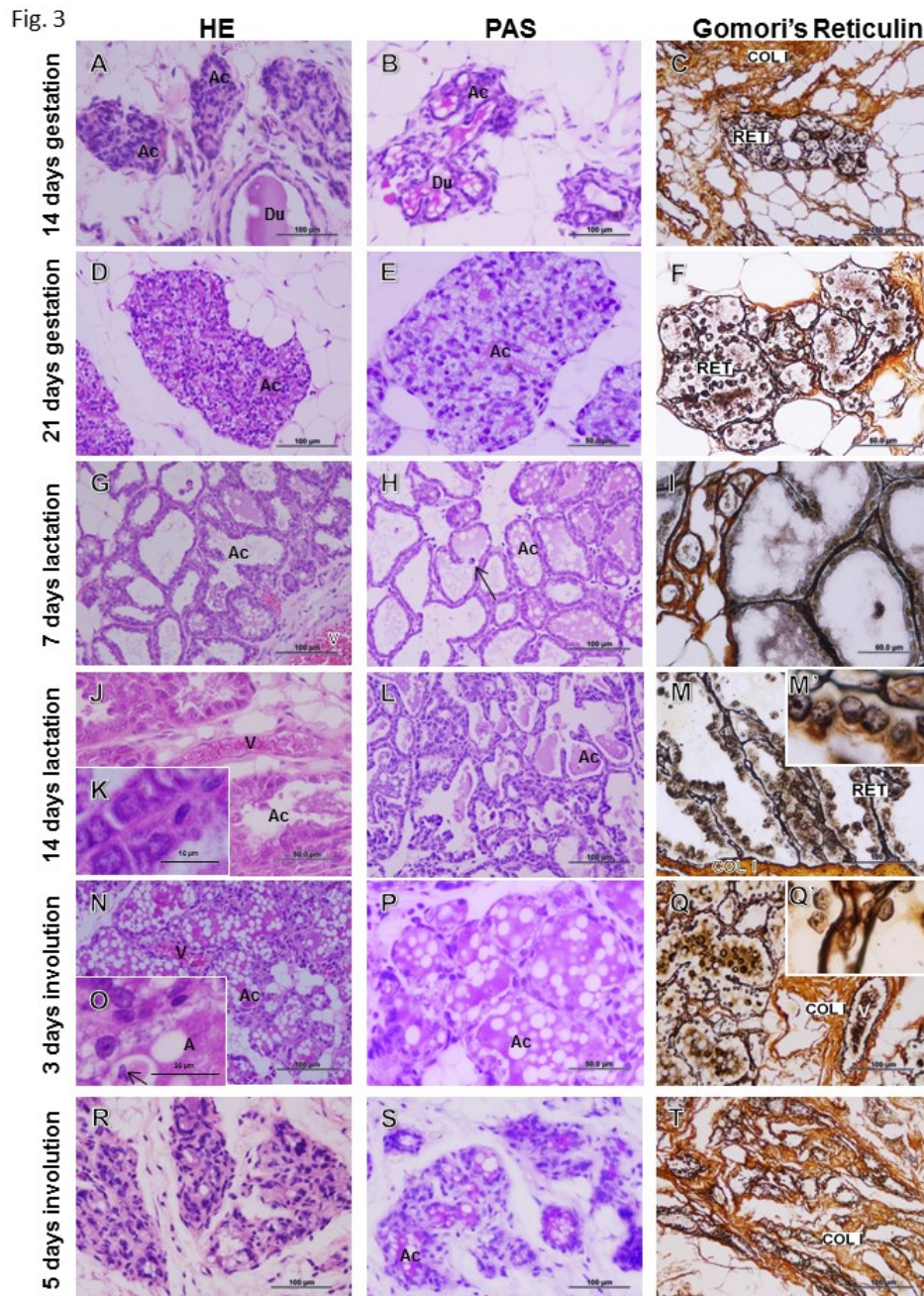


Fig. 3. Morphological aspects of the female gerbil's mammary gland stained with Haematoxylin & Eosin (HE), the Periodic Acid-Schiff (PAS) staining, and Gomori's Reticulin staining, during gestation (A–F), lactation (G–M), and involution (N–T). The acini (Ac), the ducts (Du), the blood vessels (V), and the stroma components, such as the collagen fibers Type I (stained in brown, COL I), the reticular collagen fibers Type III (stained in black, RET) and the detached epithelial cells in the acini (arrow), are shown.

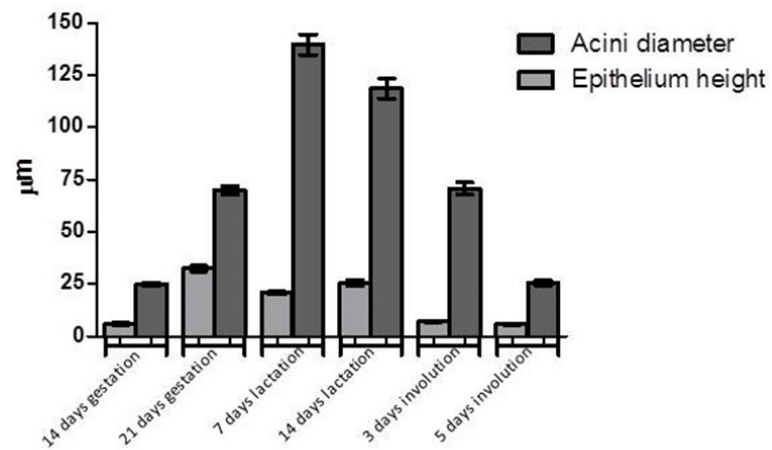


Fig. 4. The mean and standard deviations of the biometric values of the secretory portion of the female gerbil's mammary gland at different stages of the reproduction: pregnancy, lactation and involution (n = 1).

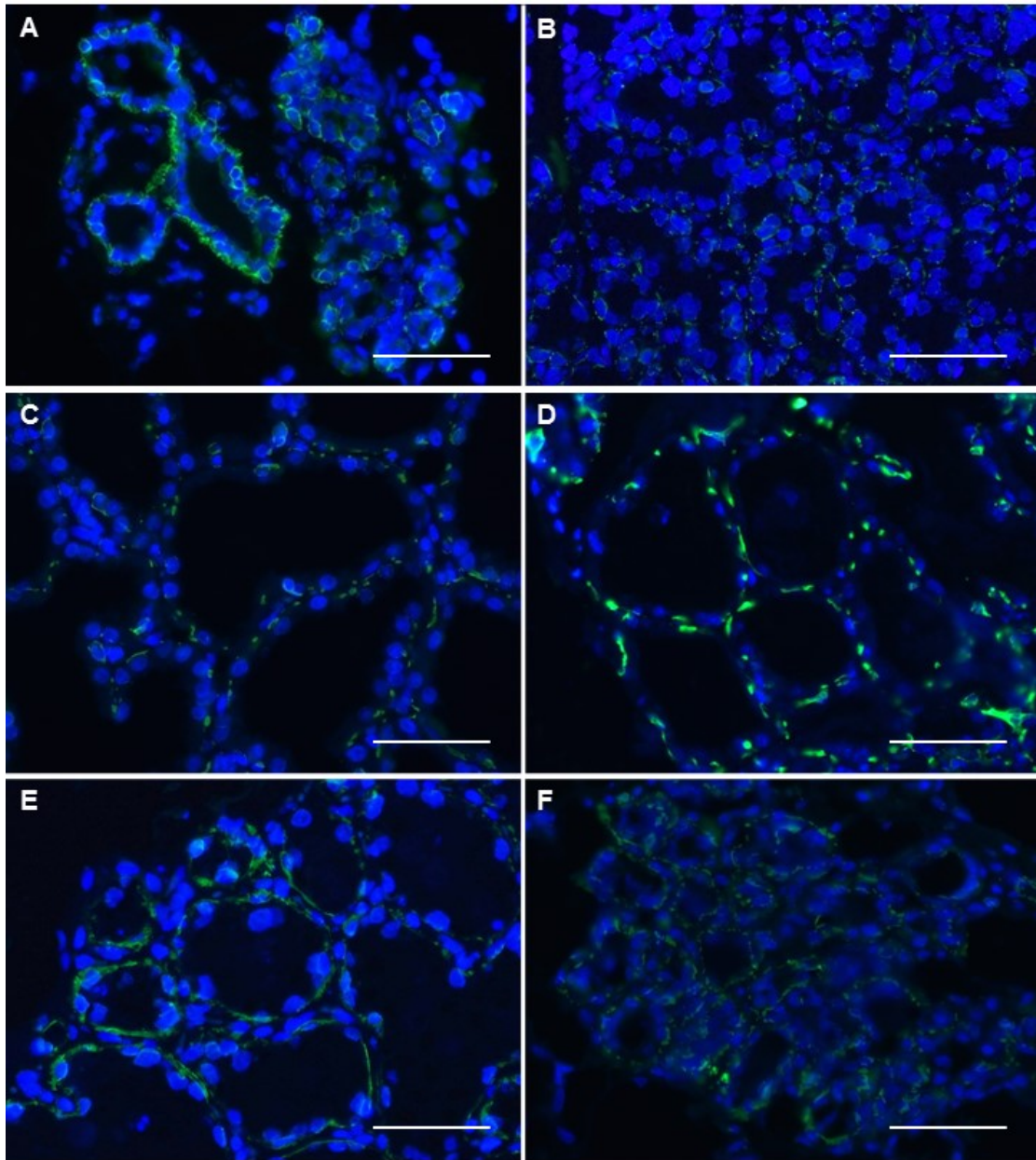


Fig. 5. Fluorescence images for the identification of the myoepithelial cells in the female gerbil's mammary gland through the immunohistochemistry for the α -actin. A: 14 days gestation; B: 21 days gestation; C: 7 days lactation; D: 14 days lactation; E: 3 days involution; F: 5 days involution. Bars: 50 μ m.

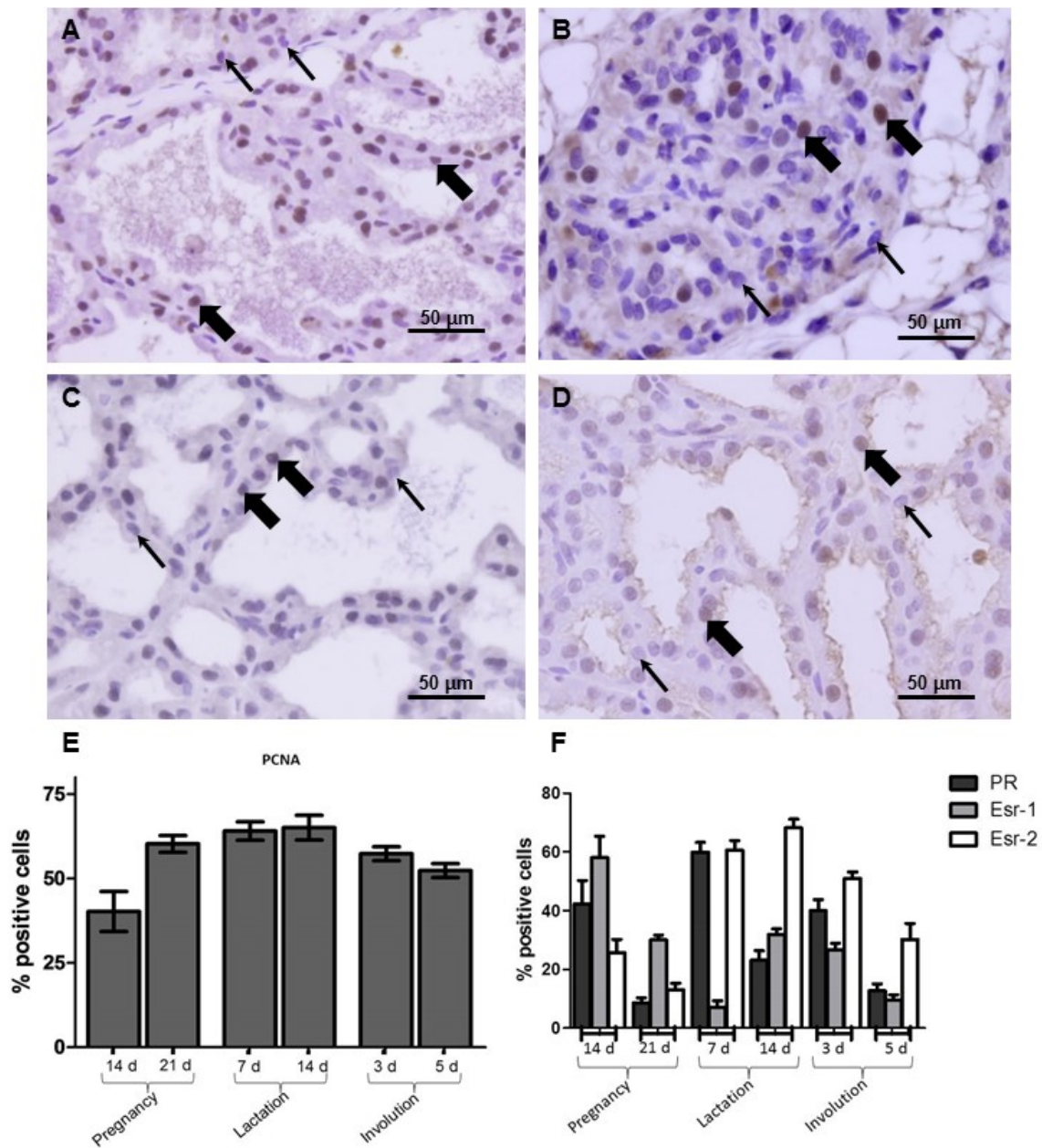
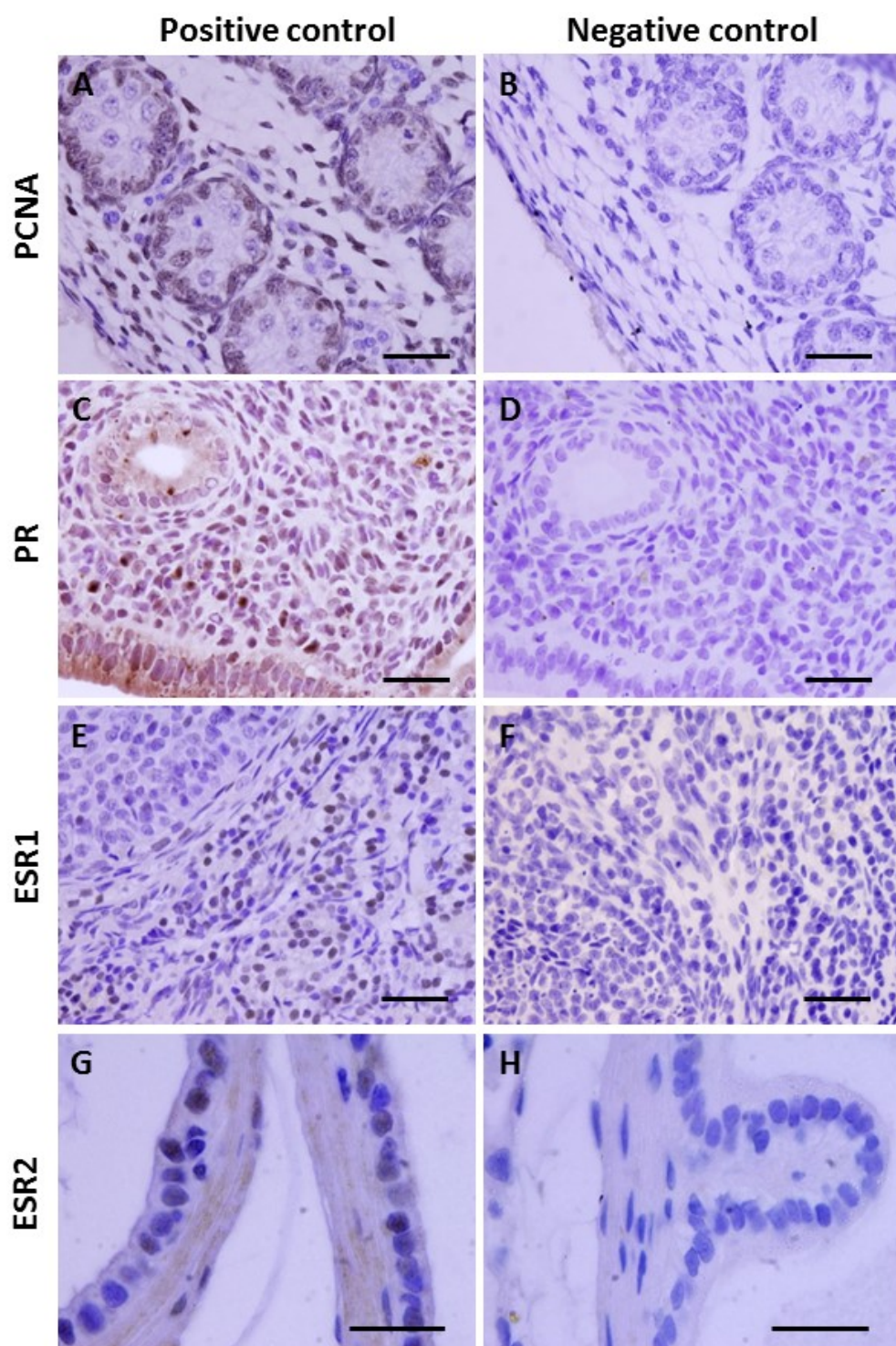


Fig. 6. Immunohistochemistry for the PCNA, the PR, the ESR1 and the ESR2 positive cell detections in the female gerbil's mammary gland. Gland marked with PCNA at gestational day 7 (A), PR at involution day 5 (B), ESR1 at lactation day 14 (C) and ESR2 at gestational day 21 (D). The figures show few positive cells (thick arrows), and negative cells (thin arrows). The percentages of PCNA (E) and the receptor positive cells (F) are also shown ($n = 1$). Bars: 50 μ m.

Table 1. Values in μm^2 of the secreting epithelium and the stroma area that were obtained in the histological sections from the female gerbil's mammary gland at different stages of the reproduction and the relationship between the parameters (n=1).

Stage	Total Area	Secreting Epithelium Area	Stroma Area	Ratio Epithelium/adipocytes and stroma
14 days gestation	30.18	6.40 (21.2%)	23.77 (78.8%)	0.26
21 days gestation	33.72	7.62 (22.6%)	26.09 (77.4%)	0.29
7 days lactation	22.25	15.70 (70.6%)	6.54 (29.4%)	2.4
14 days lactation	23.05	16.68 (72.4%)	6.36 (27.6%)	2.62
3 days involution	94.75	37.59 (39.7%)	57.16 (60.3%)	0.65
5 days involution	44.95	9.64 (21.5%)	35.3 (78.5%)	0.27



Supplementary data. The figure shows the positive and negative controls obtained from Mongolian gerbils tissues. A-B: 7 days old testicle as control for PCNA; C-D: adult uterus as control for PR; E-F: ovary as control for ESR1; G-H: prostate as control for ESR2. Bars: A-F: 50m; G-H: 20 m. PCNA: proliferating cell nuclear antigen; PR: progesterone receptor; ESR1: estrogen receptor α ; ESR2: estrogen receptor β .

5.2 Capítulo 2

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Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils

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Abstract

The endocrine disruptive effects caused by bisphenol A (BPA) are well known. Despite this, to date, evaluation of its long term effects is limited, meaning that there is still much to be unveiled in terms of alterations caused by perinatal exposure to BPA. Our aim was to determine if perinatal exposure to two different doses of BPA causes long term morphological and molecular alteration effects in the mammary gland (MG). We evaluated MG from Mongolian gerbil offspring exposed perinatally (during gestation and lactation) to 50 or 5000 µg/kg/day BPA. At 90 days of age the animals were subjected to a single dose of N-nitroso-N-methylurea in order to mimic a carcinogenic environment. At 6 months of age, animals in estrous were euthanized for morphological evaluation of the MGs. The MG architecture presented considerable changes in terms of detached epithelial cells, inflammation, glandular hyperplasia, and collagen fiber deposition, as seen by picrosirius staining. Furthermore, a higher index of epithelial cell proliferation, shown by phospho-histone H3 immunohistochemistry, was detected in comparison to the intact control group. In addition, we verified a higher molecular expression of EZH2, a marker of epigenetic alteration, in the vehicle treated group, indicating that corn oil applied alone can alter the

expression of this epigenetic biomarker. In conclusion, BPA perinatal exposure promotes significant changes in glandular cytoarchitecture and increases glandular epithelium proliferation rate, leading to the retention of stem-like properties. This event could compromise the fate and differentiation potential of mammary epithelium.

Keywords: BPA, estrogen, environment pollutant, morphologic alterations, phospho-histone-h3, EZH2

Introduction

Bisphenol A (BPA) is a compound used to manufacture plastics and resins. The main polycarbonates containing this compound are food and water storage containers. BPA residues may still be present in the epoxy resins used to make coatings inside food cans. The main concern is that BPA is released from its source to food and beverage stored in these containers ("Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs," 2015). According to the European Food and Safety Authority (EFSA), the substance is known to induce morphological and proliferative changes in the mammary gland (MG). Consequences of BPA include estrogen-like effects that may induce a behavior similar to natural 17- β estradiol (Michałowicz, 2014); even more worrisome is the fact that BPA is able to bind to estrogen receptors, avoiding endogen estrogen access (Wetherill et al., 2007) and even causing distinct agonistic/antagonistic toxic effects (Zhuang et al., 2014). Some experiments have been developed in rodents and have revealed that, even at very low concentrations, BPA may cause alterations related to hyperplasia in both male and female MGs (Mandrup et al., 2016).

During mammary organogenesis and development, estrogen signaling is critical; consequently, exposure to xenoestrogens during this period may cause lifelong negative impacts (Colborn et al., 1993). In rodents, the risk of MG tumor formation is considerably increased after in utero exposure to BPA (Durando et al., 2006). This mainly occurs because during this development period a dynamic environment takes place and the limits between epithelium and stroma with a basal membrane are ruptured (Cowin and Wysolmerski, 2010), facilitating free contact of precursor mammary epithelial cells with available BPA in the bloodstream.

In addition to nuclear receptor binding, the molecular mechanisms involved in BPA action in the reproductive tract include alterations in hormone metabolism, and genetic and

epigenetic regulation (Acconcia et al., 2015). BPA is known to decrease expression of cholesterol side-chain enzyme cleavage (CYP11A1) and steroidogenic acute regulatory protein (StAR), two rate-limiting factors for steroidogenesis, in rodent ovarian follicles and to increase the metabolism of natural hormones (Peretz and Flaws, 2013), these being important means by which this disruptor influences hormone-dependent signaling pathways.

Although altered morphological phenotypes are frequently seen in the microenvironment of exposed MGs (Hindman et al., 2017), other alterations may cause a more silent effect, consisting of epigenetic alterations such as EZH2 expression for example, that take longer to be evidenced at the morphological level but are persistent in changing gene expression (Doherty et al., 2010). These changes may modify chromatin structure and tumor suppressing regions can be methylated and lead to tumor formation (Jones and Baylin, 2002). Thus, environmental pollutants can trigger epigenetic changes in parents, that can be transferred to the offspring (Acconcia et al., 2015) and this risk has previously been linked to predisposition to diseases later in adulthood (Chiam et al., 2009).

Since mammary organogenesis requires these interactions between epithelium and stroma (Sakakura et al., 2013), the relationship between these compartments should also be explored and evaluated in terms of extracellular matrix deposition and alterations caused by BPA treatments. Many years ago the hypothesis was raised that mammary epithelial carcinogenesis arises from indirect effects of carcinogens on stroma (Steinmuller, 1971). Even in more recent years the possible roles of stroma signals have attracted interest in terms of other strands for cancer research and may consist of important targets for designing anti-cancer drugs (Sakakura et al., 2013).

The evaluation of different parameters in association is of extreme importance in terms of obtaining new knowledge regarding the pathological effects occasioned by carcinogenic compounds. Although many questions have been answered, there is still much to be explored regarding the long-term effects of BPA after in utero exposure. The use of an alternative experimental model, the Mongolian gerbil, has raised interest in the research field, suggesting an interesting species to assess the malignant effects of xenoestrogens in reproductive secreting organs such as the prostate (Facina et al., 2017; Gonçalves et al., 2017, 2013) and testes (Christante et al., 2018). Considering that endocrine disruptors have species-specific effects not yet widely explored in the MG, we decided to adopt this species

to evaluate for the first time the consequences of BPA treatment. Thus, the aim of this experiment was to evaluate the long term deleterious effects caused by exposure to different doses of BPA during the early stages of development in the MG of Mongolian gerbils.

MATERIAL AND METHODS

Animals and ethics statement

A total of 20 pregnant Mongolian gerbils were firstly used so 40 females obtained from these offspring could be analyzed in the experiment. The animals were isolated in ventilated polysulfone cages and maintained in the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP). The temperature ($24 \pm 2^\circ\text{C}$) and luminosity (12 h light/dark cycle) of the environment were controlled and all the equipment used was free from BPA, according to the manufacturer's specifications (Alesco, Monte Mor, Brazil). The animals received specific balanced feed (Presence, Paulínia, Brazil) and fresh filtered water ad libitum. The procedures were conducted according to the standards defined by the National Council of Animal Experiment Control (CONCEA) and were authorized by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP (protocol number 113/2015).

Experimental design

The experimental design is illustrated in Figure 1. Briefly, the pregnant animals were divided into four groups according to the treatment received. The first group (IC) did not receive any treatment during the period; the second group (OC) was subjected to daily treatment with 0.1 ml of corn oil (Mazola, Mairinque, Brazil) by gavage; and the third ($\downarrow$ BPA, 50 $\mu\text{g}/\text{kg}$, Vandenberg et al., 2012) and fourth ($\uparrow$ BPA, 5000 $\mu\text{g}/\text{kg}$) groups received daily doses of BPA also by gavage diluted in 0.1 ml of corn oil. The manipulations were performed from the 8th day of gestation until the last day of lactation (total 46 days of treatment). The treatment was suspended only on the parturition day. On each day the gavage was performed during the morning.

After weaning, two females obtained from each offspring were used for future analysis; other individuals obtained in the same family were used in other experiments. The selected females were maintained under the previously described conditions until 90 days of age. At that point, half them received one single intraperitoneal application of N-nitroso-N-methylurea – NMU (1517, Sigma Aldrich St. Louis, USA); the other female from the same

family was kept as a negative control for this treatment. After 90 further days, all animals were euthanized by stunning induced with CO₂ followed by decapitation. The procedures were performed during the morning with the animals always in the estrous phase of the cycle.

Histological processing and cytochemical staining

During the euthanasia procedures, the weight of the whole body, liver, and ovaries + uterus complex were evaluated. These organs were also evaluated for the possible presence of macroscopic lesions. The MGs were collected for analysis. The right abdominal gland was removed carefully and immediately fixed with paraformaldehyde 4% for 24 hours. Next, the samples were transferred to alcohol 70% and histologically processed under a semi enclosed system (TP1020, Leica Biosystems, Buffalo Grove, USA). The paraffin blocks were sliced (4 µm thick) and slides were mounted. Cytochemical staining was performed with hematoxylin and eosin (HE) as well as with picosirius, to evaluate the morphological alterations in tissues from different groups.

For the picosirius technique, the slides were dehydrated and stained for 1h in Sirius Red solution (Direct Red 80 diluted in saturated picric acid solution). After washing, the slides were counterstained with hematoxylin for 10 minutes, dehydrated, and mounted.

Analysis and quantification of histopathological alterations

The incidence and multiplicity of lesions were evaluated in all animals. Three sections from different depths of each block were stained with HE and picosirius (n=5) and digitalized at 400x magnification using a BX61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120 Virtual Microscope Slide Scanning System (VS120-S5) from the same company.

Since neither malignant nor premalignant lesions have been previously described in the MG of Mongolian gerbils, the bases for our classification were publications describing these alterations in rats and mice (Medina, 2008; Rudmann et al., 2012; Russo et al., 2000; Singh et al., 2000). Tissue sections were evaluated blindly according to the experimental group. The incidence of lumen with leukocytes or detached epithelial cells, as well as inflammation foci and hyperplasia were evaluated.

The picosirius stained slides were analyzed in a light microscope coupled to a polarized light, which allowed identification of the birefringence of collagen fibers due to their different molecular composition. Thirty random microscopic fields per group were acquired

and evaluated according to the birefringence spectrum. Stereological analysis was carried out to determine the frequency of collagen patterns according to the M130 multipoint test system (Weibel, 1963). Points coinciding with areas of yellow or red, epithelial cells or stroma were counted, for calculation of the frequency as a percentage over the total counted points. This analysis aimed to estimate the deposition of collagen fibers in the secreting structures of the MG.

Immunohistochemistry

The primary antibodies used were anti- α -actin (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology); anti-phospho-histone H3 (P-H-H3, rabbit polyclonal, H3, 1:75, Ser10, 9701, Cell Signaling, Danvers, USA); and anti-enhancer of zeste homolog 2 (EZH2, rabbit monoclonal, 5246s, 1:100, Cell Signaling, Danvers, USA). Endogen peroxidases were blocked by immersing the slides in 3% H_2O_2 at room temperature for 30 minutes. Antigen retrieval was performed by immersing the tissue sections in 10mM citrate buffer pH 6.0; the temperature and time for retrieval were 96°C for 40 minutes for P-H-H3, 93°C for 20 minutes for α -actin, and 98°C for 75 minutes for EZH2. Next, nonspecific proteins were blocked using 10% normal goat serum + 1% bovine serum albumin (BSA) in TBS for EZH2, 5% BSA in TBS for P-H-H3, and 5% skimmed milk in PBS for α -actin. The sections were incubated overnight at 4°C (P-H-H3 and EZH2) or for 1h at room temperature (α -actin) before secondary antibodies (EZH2:Envision anti-rabbit, HRP, K4003, DAKO-Agilent, Santa Clara, USA; P-H-H3 and α -actin: Polymer kit Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) were applied for 1h at room temperature. Subsequently, positive staining was detected using 3-30'-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstained with hematoxylin.

Determination of α -actin expression, cell proliferation index, and EZH2 positive cells

For α -actin, thirty random macroscopic fields were digitalized at 400x magnification and stereological analysis was carried out to determine the frequency of myoepithelial cells according to the M130 multipoint test system.

Thirty microscopic fields per group (n=5) were analyzed to determine the relative frequency of P-H-H3 and EZH2 positive cells in MG tissue. The cell proliferation index and percentages of EZH2-positive cells were calculated by dividing the number of positive nuclei by the total number of nuclei counted. The results are expressed as percentages.

Protein expression quantification

The protein content of the left abdominal MG sample was extracted with a lysis buffer according to the protocol described by Li et al (2009); the protease activity was blocked by adding an inhibitor. Samples were smashed and left for extraction at 4°C for 1h under agitation, before being centrifuged for 20 minutes at 14000 rpm at 4°C. The extracts were stored at -80°C until analysis. For quantification, a BCA Protein Assay Kit (23227 Thermo Fischer) was used and the absorbance was read in an absorbance microplate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany).

EZH2 was quantified in MG samples by Western blotting. A total protein amount of 15 µg was placed in each well in the SDS page gel and subjected to electrophoresis (120 V for 10 minutes then 170 for 30 minutes). The bands were transferred to a PVDF membrane (100 V for 1 hour). Blots were blocked using BSA 5% in TBS + 0.1% Tween (TBSt) for 1 hour at room temperature. Overnight incubation with anti-EZH2 (rabbit monoclonal 5246s Cell Signaling, Danvers, MA, USA, 1:1000) and GAPDH (mouse monoclonal MB374 Millipore, Burlington, MA, USA, 1:10000) diluted in BSA 1% in TBSt was performed. Then the membranes were washed in TBSt and incubated at room temperature for 1 hour under agitation with horseradish peroxidase conjugated secondary antibodies (goat anti-mouse 115-035-062 or goat anti-rabbit 115-035-144, Jackson ImmunoResearch, West Grove, PA, USA, 1:10000). After washing, the membrane antibody detection was revealed with ECL Substrate Pierce (32109 ThermoFischer, Rockford, IL, USA) and Super Signal (34094 ThermoFischer, Rockford, IL, USA) reagents. The membranes were revealed in a Fusion machine (Fusion Solo VilberLourmat, Marne-la-Vallée, France) and the band densities were analyzed in a densitometry program – Image J (version 1.52a, Wayne Rasband, NIH, USA).

Statistical analysis

The statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad 5.0, Inc, CA, USA). The quantitative analysis was based on parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis followed by Tukey's or Dunn's multiple comparison tests, respectively. Results were considered significant at the $p < 0.05$ level.

RESULTS

Histopathological alterations

Evaluation of the animals during the maintenance period did not reveal the presence of any macroscopic lesions in the regions of the MGs when palpation was performed. During

euthanasia procedures, no macroscopic lesions in the organs were found and there were no differences between the groups regarding the weights obtained; the presence of a swollen uterus and some cysts in the ovaries being the only noteworthy alteration observed in some cases.

The incidence of morphological alterations showed some significant differences between the evaluated groups (Figure 2). The values are shown in Table 1 provided as supplementary material. In summary, the highest values were found in the ↓BPA group, where a higher incidence of leukocytes was present in comparison to the OC and ↑BPA groups (Figure 2A). The incidence of detached epithelial cells was also higher in the ↓BPA group without NMU in comparison to all other groups non-treated with NMU (Figure 2C).

Signs of inflammation were characterized by the presence of inflammatory cells among the conjunctive tissue or through the epithelial structures (Figure 2G-H). This was more frequent in the ↓BPA group non-treated with NMU in comparison to both the IC groups.

Hyperplasia was characterized by the presence of two or more layers of epithelial cells in the acini or ducts, sometimes covering the totality of the structure (Figure 2J-K). Its incidence differed between the ↑BPA and IC groups and was also higher in the ↑BPA and ↓BPA groups in comparison to the OC.

The results showed that, with the exception of hyperplasia in the OC group and inflammation in the ↓BPA group, no differences were found between animals treated or not with NMU regarding morphological alterations. This led us to perform the remaining evaluations only in animals from the control subgroup (not treated with this compound) to evaluate the effects caused by each treatment.

The deposition of collagen in the stroma and among the adipose tissue was quantified and is shown in Figure 3. A higher incidence of collagen was found in the IC (21.5 ± 2.8) in comparison with the OC (12.5 ± 0.7) and ↑BPA (13.4 ± 0.5) groups. The ↓BPA group (18.7 ± 1.9) did not differ from any other group.

Expression of α -actin

The maintenance of morphology in the secretory units was complemented with α -actin identification by means of immunohistochemistry. As expected, the myoepithelial cells were present in the compartment surrounding the basal lamina; no staining was found in the other compartments. The analysis showed that, in general, the structures were well preserved and with the myoepithelial layer maintained, therefore there were no differences

between the groups regarding the presence of this protein in the tissue evaluated. Illustrative pictures and the quantification of α -actin are shown in Figure 4.

Cell proliferation index

The quantification of positive cells for P-H-H3 revealed that all groups treated with BPA demonstrated increased proliferation indices in comparison with the intact control group. The means $\pm$ standard error as well as illustrative pictures are shown in Figure 5.

Expression of EZH2: immunohistochemistry and Western Blotting

The quantifications and illustrative photomicrographs of EZH2 are shown in Figure 6. Although a slight increase in positive cells was observed in the OC group, the statistical analysis did not show any differences between the treatments in terms of positive cells for EZH2 (Figure 6A). This led us to go further through the analysis and evaluate the total amount of this protein in the extracts obtained from the samples through western blotting. The results demonstrated that the amount of protein expressed in the MGs from the group treated with oil was significantly higher than the intact control group.

DISCUSSION

The results described in the present study showed that perinatal exposure to 50 or 5000 $\mu\text{g/kg}$ of BPA induced an increase in the incidence of morphological alterations in the MGs after 6 months of age: detached epithelial cells and leukocytes in lumen, inflammation, collagen deposition, and hyperplasia modifications were described. However, the gland architecture as observed through myoepithelial cell presence and organization was not altered in any of the groups. A prominent difference, however, was present in the proliferation index of both BPA treated groups in comparison to the intact control group. Another interesting finding was the increase in EZH2 expression in the OC group, suggesting that treatment with the vehicle usually adopted for dilution and administration of liposoluble compounds increases the risk of breast cell malignancy and cancer development (Doherty et al., 2010).

The presence of detached cells was high in the $\downarrow$ BPA and low in the OC group (both without NMU). Although the presence of detached cells indicates that apoptosis is taking place in the gland – according to the morphology – the accumulation in lumen may indicate deregulations related to malignant progression (Green and Streuli, 2004). The main mechanism for their removal is phagocytosis by macrophage or neighbor cells (Gardai et al.,

2006; Monks et al., 2008), which is in accordance with the increased presence of leukocytes in the MG lumen of the ↓BPA group.

An important rise in the incidence of leukocytes in lumen was present in the ↓BPA group, with or without NMU. Immune system cells are known to protect tissue against pre-malignant alterations, destroying cells and suppressing tumor growth (Hanahan and Coussens, 2012). The higher incidence of lumen with leukocytes could indicate that cells from the immune system may have been recruited, aiming to control a possible tumorigenic environment, controlling the presence of luminal apoptotic epithelial cells, for example. Similarly, inflammation was more frequently present in the ↓BPA group in comparison to the IC. This corroborates the results of Fischer et al (2016) who stated that mice perinatally treated with this compound demonstrated deregulation of inflammatory cytokines that may lead to disordered cell growth (Fischer et al., 2016). The authors suggest that this may occur due to changes in the immune response targeting cancer cells.

As shown in Figure 2, hyperplasia was the most frequent alteration found. This lesion is considered a sentinel sign of malignancy, since the proliferation of epithelial cells, sometimes filling the lumen, is the earliest sign of a ductal or lobular carcinoma in situ (Keely, 2011; Singh et al., 2000). This alteration occurs due to a reduction in the apoptotic levels of ductal cells that may be related to reduced pro-apoptotic factors (Mailleux et al., 2007). This corroborates the previously proposed hypothesis of an apoptotic/phagocytic unbalance, possibly leading to malignant progression.

The ability of perinatally administered BPA to cause hyperplasia leading to ductal carcinoma has already been seen in Wistar rats (Gomez et al., 2017). In that experiment the authors described the presence of a typical lobular hyperplasia in a higher incidence after treatment with lower concentrations (0.5 µg/kg/day) versus high concentrations (50µg/kg/day) of BPA. Similarly to rats (Soto et al., 2008) other species, such as mice (Vandenberg et al., 2008) and rhesus monkeys (Tharp et al., 2012), showed proliferative perturbations in the adult MG after BPA perinatal exposure. In the present study, the main differences were present between the ↑BPA with NMU and control groups without NMU (IC and OC), suggesting that although the ↓BPA group showed higher impacts regarding other morphological alterations, a larger hyperplastic response was given by the 5000 µg/day exposure to BPA.

Although the majority of experimental groups demonstrated higher means of hyperplasia incidence after treatment with NMU, only the OC group presented a significant difference. In

the same way, the incidence of inflammation was higher only in the ↓BPA group without NMU in comparison to the non-NMU-treated group. Since previously published experiments described the presence of malignancy and tumors in Mongolian gerbils after NMU intraperitoneal administration (Gonçalves et al., 2013, 2010; Quintar et al., 2017), we expected the presence of neoplasias in this experiment. However, as previously described experiments did not test the use of NMU in female gerbils we hypothesize that in this case the time necessary to develop mammary neoplasia is longer; female rats (Cha et al., 1994) usually show macroscopic lesions after 90 days of NMU administration. Nevertheless, it is known that susceptibility to NMU carcinogenesis may vary according to species (Medina, 2007) and that mice do not develop mammary tumors unless hormonally stimulated; therefore, the hormonal milieu of the time of NMU treatment influences most the incidence and type of tumor (Guzman et al., 1992). Another hypothesis is that perinatal exposure to BPA induces a marked action, in terms of morphological alteration, during specific hormonal stages such as gestation and lactation. These findings suggest that, in Mongolian gerbils, the development of female mammary tumors requires more time to occur or a higher dose of NMU.

The absence of substantial differences after NMU treatment and higher lesion incidences in the BPA treated groups led us to focus attention on the effects caused only by this hormonal disruptor. Other morphological and molecular markers were then evaluated exclusively in the animals not exposed to NMU.

The deposition of collagen fibers in the stroma indicates that the state of the architecture and function of the gland and extracellular matrix (ECM) remodeling is directly related to the proliferative and invasive state of tumors (Keely, 2011). This is the reason why so much importance is given to periodic mammographic examinations in adult women (Huo et al., 2015). We chose picrosirius staining, which gives red/yellow birefringence according to the crosslinking stage of the collagen fibers. Our results showed higher percentages of collagen deposition in the ECM of the IC group, probably meaning that the capacity of ECM to maintain its function of tumor suppression was decreased in the OC and ↑BPA groups. On the other hand, the gland architecture in terms of the basal membrane was maintained, as shown by α -actin marking and no differences in its expression index were found between the groups. According to Thompson et al (Thompson et al., 2017), there is a higher expression of α -actin in the MG of caveolin-1 knockout mice, a protein that has been linked

to tumor aggressiveness; in addition to which the expression of collagens was upregulated. The authors, though, justify this finding by the presence of additional ducts in the knockout model and not an increase in the thickness of the myoepithelial layer. For us the absence of an aberrant ECM may be a factor that contributed to avoid tumor progression since an adequate environment for tumor initiation was not present.

BPA is known for its developmental disturbance and the majority of the alterations are occasioned by its estrogen-agonist activities. It is probable that this is the reason why the significant result obtained referred to the higher incidence of proliferative cells in the treated groups, as shown by the P-H-H3 quantification in the epithelial mammary structures. The phosphorylation of histone H3 is directly linked to cell division (Hans and Dimitrov, 2001). Histones are fundamental compounds that contribute to build nucleosomes (Arents et al., 1991) and they phosphorylate both during transcriptional activation, when chromatin is uncondensed (Nowak and Corces, 2000), and during cell division with a compacted chromatin (de la Barre et al., 2000). Thus, P-H-H3 becomes a valuable tool to quantify cell proliferation since phosphorylation is detected in the heterochromatin at the late G2 phase and spreads through all chromosomes as mitoses develops (Hans and Dimitrov, 2001). This has been successfully adopted as a proliferation marker (Chow et al., 2017; Elmaci et al., 2017). It has previously been stated that exposure to xenoestrogens during fetal development induces alterations that generate altered control of cell proliferation (Durando et al., 2006); thus, our results corroborate the hypothesis that perinatal exposure to BPA increases the sensitivity of the MG to endogenous estradiol, creating a permissive situation that facilitates malignancy (Muñoz-de-Toro et al., 2005).

Finally a last factor that raised interest in terms of MG alteration was the expression of EZH2 in the parenchyma. Epigenetic regulation produces heritable changes, as well in gene expression. EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), a histone methyltransferase involved in tumorigenesis (Doherty et al., 2010). In this way, EZH2 is able to provide the methyltransferase activity resulting in the transcriptional silencing of target genes (Gulati et al., 2018). Mice with overexpression of EZH2 developed a disorganized ductal epithelium (Li et al., 2009). EZH2 knockout mice present a delay in ductal elongation during puberty and in lobuloalveolar expansion in pregnancy (Michalak et al., 2013). No differences were found between the groups in the immunohistochemistry analysis; however, we verified that gestational and lactational exposure to corn oil caused a

significant increase in the expression of EZH2, as evidenced by western blotting. This oil has been widely applied as a vehicle for administration of lipophilic compounds. Previously described experiments showed that gavage administration of corn oil produced elevated corticosterone levels in comparison to methylcellulose, Tween, or water used as vehicles (Brown et al., 2000). The authors add that administration with lipid vehicles induces activation of a stress response. We believe that there may be an interaction between BPA and the vehicle used, sometimes causing an alteration in the usual effects. A recent publication has described the protective effect in the liver when sesame oil is used for BPA administration (Elhamalawy et al., 2018). Corn oil is known to cause alterations in gene expression profiles in other organs, while no histopathological and cell counting alterations are seen (Geng et al., 2012). In this way, there is a possibility that the corn oil alone caused some alterations in EZH2 gene expression that did not reflect in other parameters evaluated. The importance of evaluating the effects of BPA in experimental and clinical assays consists of raising attention turned towards this compound, aiming to reduce more and more the permitted limits of use in industries. Even though nowadays very low concentrations are present in plastic compounds, there are still high plasma and serum levels in the human population all over the world (Thoene et al., 2018). The United States Environmental Protection Agency (EPA) stated that BPA has the third priority place in terms of toxicity (Reif et al., 2010). Thus the more information available regarding the long term effects of this compound in the body, the more attention the population will give this subject.

Our findings demonstrated that Mongolian gerbils showed considerable morphological alterations in MGs after perinatal exposure to BPA; furthermore, an increase was observed in the proliferation index, which could lead to the retention of stem-like properties. This event could compromise the fate and differentiation potential of mammary epithelium. In addition, we verified a higher molecular expression of EZH2 in the vehicle treated group, indicating that corn oil applied alone can also alter the expression of this epigenetic biomarker. Thus, the presence of BPA in the microenvironment of perinatal development negatively affects the architecture of MG and corn oil alone is a bigger threat in terms of epigenetic alterations in mammary tissue.

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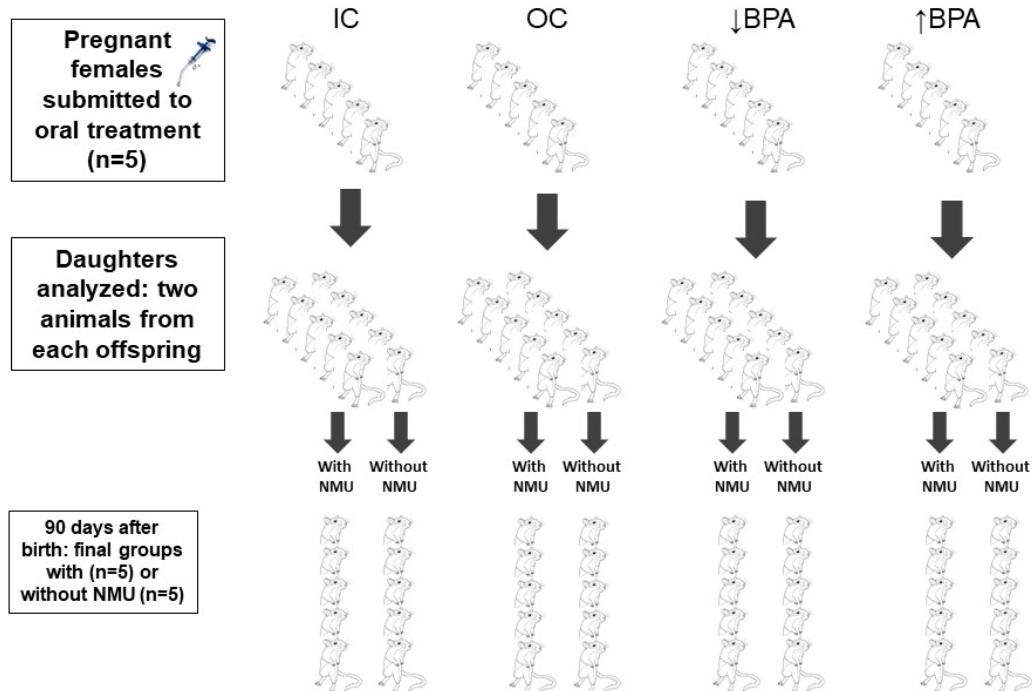


Figure 1. Experimental design. Pregnant females were left as intact control (IC), or subjected to corn oil (OC), 50 µg/kg BPA (↓BPA), or 5000 µg/kg BPA (↑BPA) daily treatments from the 8th day of gestation until the end of lactation. At 90 days of age the females from offspring obtained were divided into two groups and half treated with a single dose of NMU. The animals were euthanized at 180 days of age. BPA: bisphenol A; NMU: N-nitroso-N-methylurea.

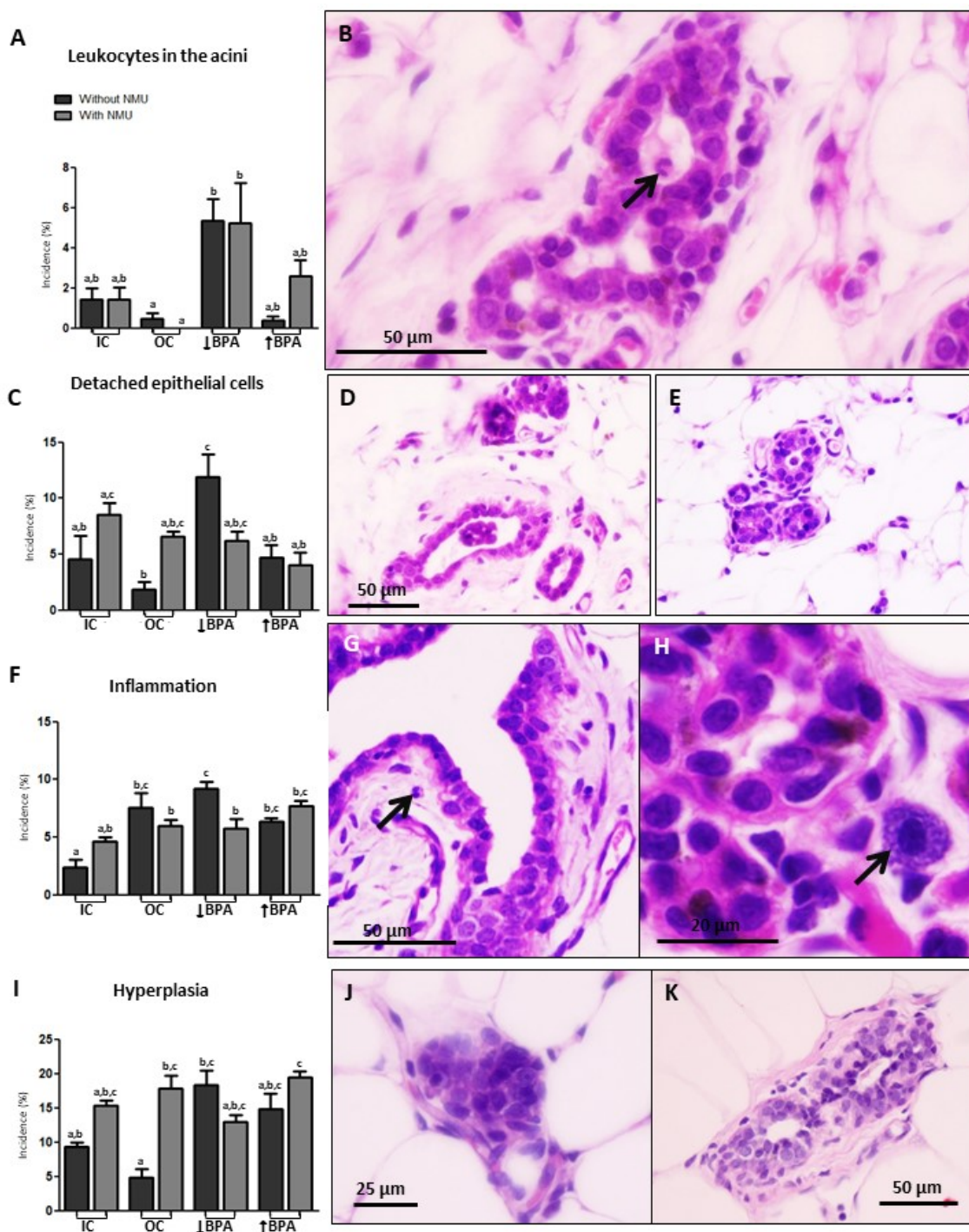


Figure 2. Incidence of morphological alterations (mean±SEM) observed in the mammary glands. Same letters in A,C,F, and I indicate similar results. Parametric (A,D,F: ANOVA followed by Tukey's test) and non-parametric values (I: Kruskal-Wallis followed by Dunn's multiple comparison test) were considered significant when $p < 0.05$. HE staining revealed presence of leukocytes (B, arrow) and epithelial cells (D-E) released in the lumen, presence of inflammatory cells among the stroma (G-H, arrows) and presence of two or more layers of epithelial cells in acini and ducts (J-K) were quantified among the secretory structures of the mammary gland.

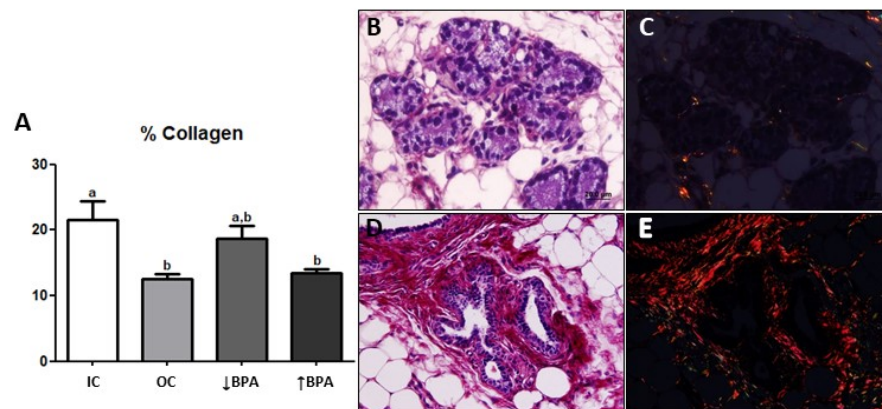


Figure 3. Percentages (mean±SEM) of collagen deposition incidence in the mammary gland of animals evaluated in different groups and pictures of mammary gland stained with picrosirius: groups OC (B-C) and IC (D-E) without NMU. ANOVA followed by Tukey's Multiple Comparison test, $p<0.05$. Bar: 20 μ m.

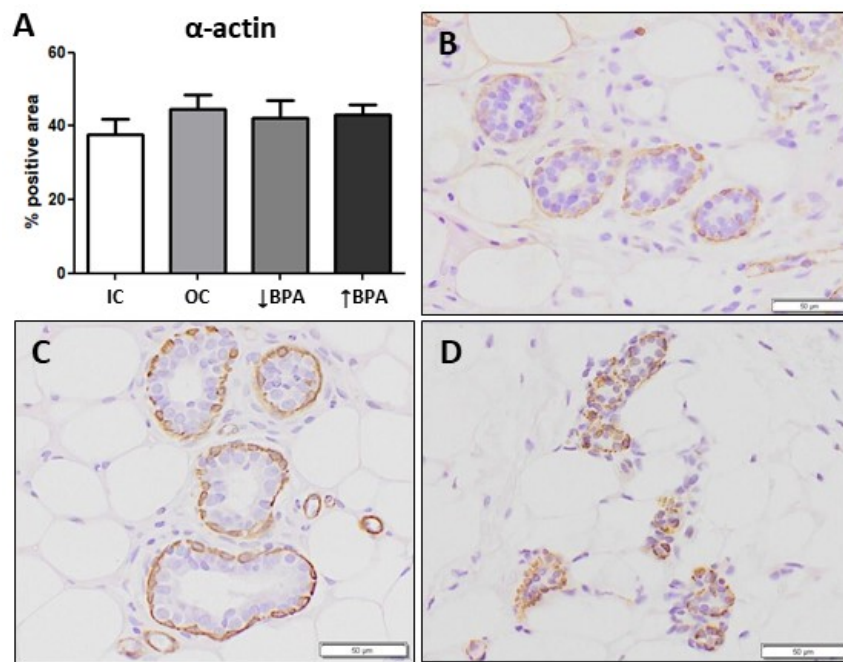


Figure 4. Percentages (mean±SEM) of α -actin expression incidence in the epithelium of mammary tissue of animals evaluated in different groups. Pictures from groups ↓BPA (B), ↑BPA (D), and IC (C) without NMU. ANOVA followed by Tukey's Multiple Comparison test, $p < 0.05$. Bars: 50 μ m.

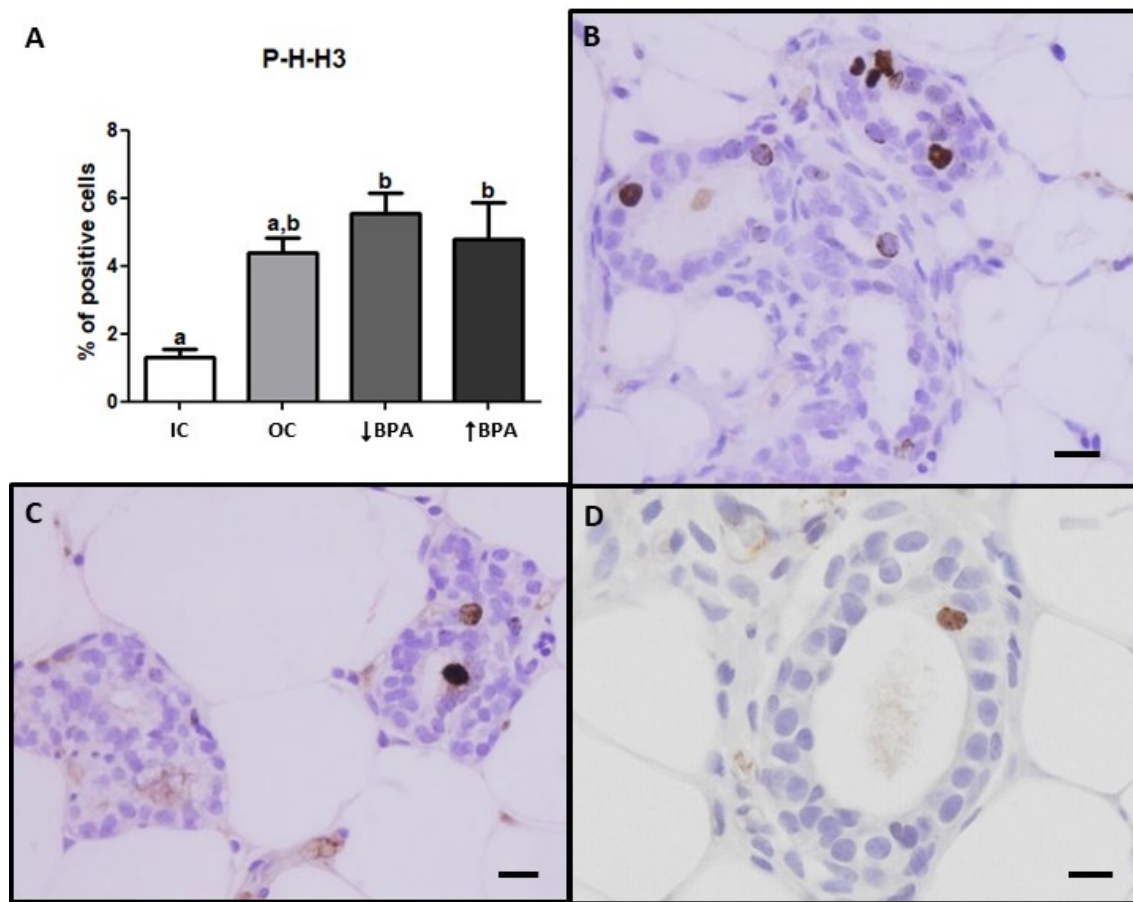


Figure 5. Percentages of positive cells for phospho histone-H3 (P-H-H3) in the different groups (A). There was a higher incidence of positive cells in the ↓BPA group (B) followed by the ↑BPA (C) and intact control (D) groups without NMU. ANOVA followed by Tukey's Multiple Comparison test, $p < 0.05$. Bars: 50 μm .

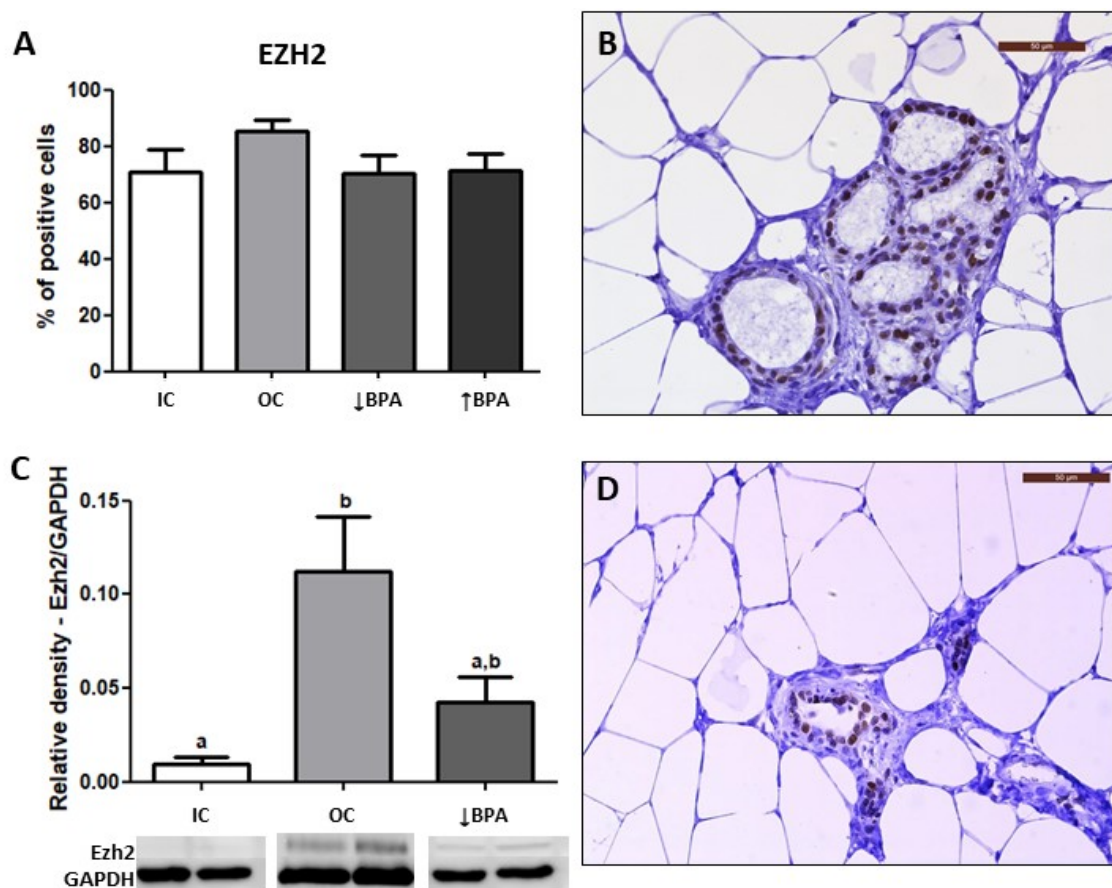


Figure 6. Percentages of positive cells (A), illustrative pictures of groups OC (B) and IC (D) without NMU, and relative density (C) of EZH2 in the mammary gland tissue evaluated after the different treatments. Different letters represent statistically different results. ANOVA followed by Tukey's Multiple Comparison test ($p < 0.05$). Bars: 50 μ m.

Supplementary Table 1. Incidences (Mean±SEM) of histopathological alterations found in the evaluated mammary glands.

Group	Leukocytes in the acini	Detached epithelial cells	Inflammation	Hyperplasia
IC without NMU	1,4±0,5 ^{a,b}	4,5±2,0 ^{a,b}	2,3±0,7 ^a	9,3±0,6 ^{a,b}
IC with NMU	1,4±0,5 ^{a,b}	8,5±1,0 ^{a,c}	4,5±0,4 ^{a,b}	15,3±0,7 ^{a,b,c}
OC without NMU	0,4±0,2 ^a	1,8±0,61 ^b	7,5±1,2 ^{b,c}	4,8±1,2 ^a
OC with NMU	0,0±0,0 ^a	6,5±0,43 ^{a,b,c}	5,9±0,54 ^b	17,8±1,9 ^{b,c}
↓BPA without NMU	5,3±1,0 ^b	11,8±2,0 ^c	9,2±0,6 ^c	18,2±2,2 ^{b,c}
↓BPA with NMU	5,2±2,0 ^b	6,1±0,8 ^{a,b,c}	5,7±0,7 ^b	12,9±1,0 ^{a,b,c}
↑BPA without NMU	0,3±0,2 ^{a,b}	4,6±1,1 ^{a,b}	6,3±0,3 ^{b,c}	14,8±2,1 ^{a,b,c}
↑BPA with NMU	2,5±0,8 ^{a,b}	4,0±1,0 ^{a,b}	7,7±0,4 ^{b,c}	19,4±0,8 ^c

^{a,b,c} Similar letters in the same column indicate statistically similar results.

5.3 Capítulo 3

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Impact of neonatal bisphenol A and 17 β estradiol exposure: comparing hormone receptors feedback

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Keywords: mammary gland, Mongolian gerbil, estrogen, xenoestrogen, pollutant

Declarations of interest: none.

Highlights:

- Perinatal exposure to BPA increases expression of progesterone receptor.
- 17 β estradiol increases expression of ER α in adult mammary gland.
- Serum estradiol decreases in oil, 17 β estradiol and BPA exposed animals.
- Perinatal BPA and 17 β estradiol modulates hormonal pathways in adulthood.

Abstract

The hormonal regulation prevails in mammary gland (MG) development. Therefore some factors can disrupt the early phases of its development, making the gland more susceptible to long term modifications of its response to circulating hormones. Endocrine disruptors, such as bisphenol A (BPA), are able to cause alterations on hormone receptor expression, leading to changes on cell proliferation index that may expose the tissue to neoplastic alterations. Thus we evaluated the variations of hormone receptors expression in the MG of 6 month old Mongolian gerbils exposed to 17β estradiol (G3) and BPA (G4) during the perinatal period; intact control (G1) and vehicle control (G2) groups were evaluated as well. Receptors for estrogen alpha ($ER\alpha$), beta ($ER\beta$), progesterone (PGR), prolactin (PRL-R) and co-localization of connexin 43 (Cx43) and $ER\alpha$ in gerbils were analyzed; serum concentrations of estradiol and progesterone were assessed as well. No alterations in body, liver and ovary + uterus complex weights were observed. However, there was an increase of epithelial $ER\alpha$ expression in the G3 group and of PGR in the G4 group. Despite $ER\beta$ did not show alterations by immunohistochemistry analysis, western blotting revealed an intense decrease of this protein in the BPA group. PRL-R was more present in epithelial cells in G2, G3 and G4 groups in comparison to G1. Cx43 was more frequent in G3 and G4 groups, suggesting a protective response from the gland against possible malignancy. Serum concentration of estradiol reduced in G2, G3 and G4, confirming that alterations in the steroidogenic rate also happened. Based on that, perinatal exposure to BPA and the reference endogen estrogen, 17β estradiol, are able to change the susceptibility of MG to endocrine disruption in a long term manner, since repercussions are observed even at 6 months after the exposure.

1. Introduction

The rise in human innovation and, consequently industrialization demands technology adaptations. The development of practical materials, such as plastic, was revolutionary and its benefits are undeniable for different areas such as industry, medical compounds, and commodities. The manufacturing of plastic however must be rethought, according to the UN World Environment Day Outlook 2018, since huge amounts of plasticizers, elements added to polymeric materials that assist its processing and formulation (Pereira et al., 2015) have been applied. The widely adopted compound bisphenol A (BPA) is usually chosen for manufacture of plastic and synthesis of epoxy resins (Andrady and Neal, 2009; Hoekstra and Simoneau, 2013).

BPA is known as an endocrine disruptor due to its capacity to mimic the effects of endogenous hormones (NIH, 2018). It is also classified as a xenoestrogen, due to its capacity to bind to estrogen receptors (ERs) (Wetherill et al., 2007). Because of its structure, BPA is able to bind both ER α and ER β . Since these are transcription factors, after ligand binding these receptors migrate to the nucleus aiming to regulate the expression of estrogen target genes (Ascenzi et al., 2006). However the post binding conformation of ERs may change according to the ligand, in a way that xenoestrogens may promote an “inappropriate” binding (Acconcia et al., 2015). These receptors play an important role in growth and maintenance of a variety of organs and tissues: mammary gland (MG), uterus, bone, heart (Delfosse et al., 2014). Despite they may be expressed separately in some organs, in the MG both are present and exert different functions. With that, the proliferative and anti-proliferative effects of ER α and ER β , respectively, may be disturbed due to impacts in the balance between both receptor functions (Acconcia et al., 2015). Besides binding to ERs, BPA also exerts impacts in the expression of these receptors (Somogyi et al., 2016).

In addition, progesterone (PGR) (Krishnan et al., 1993; Li et al., 2016; Muñoz-de-Toro et al., 2005), androgen (Delfosse et al., 2012), growth hormone (Dang et al., 2009) and thyroid hormone receptors (Somogyi et al., 2016) may also be disrupted due to BPA exposure. Some PGR dependent uterine functions have shown to be altered after prolonged BPA exposure (Li et al., 2016), affecting embryo implantation and decidualization during early pregnancy. In rats perinatally treated with BPA, increased induction of hormone function key mediators was observed (Durando et al., 2011). Prolactin release has also shown to be altered by BPA, since it mimics estradiol in inducing hyperprolactinemia due to its binding to ER in the pituitaries (Steinmetz et al., 1997).

It was confirmed that even when xenoestrogens exposure happens in the first stages of development, long term effects are present, being the adult individuals still vulnerable to the consequences of perinatal BPA (Durando et al., 2006; Muñoz-de-Toro et al., 2005). This effect is known as estrogenic imprint due to long term effects of estrogen exposure (Perez et al., 2016; Prins et al., 2007). Despite acting by different pathways, 17β estradiol has a more potent effect since its estrogenic activity at a concentration of 10^{-8} M is similar to that of BPA in a concentration of 10^{-6} M (Hiroi et al., 1999). Thus, the aim of this work was to verify the influence of perinatal BPA and 17β estradiol, used as a reference endogen estrogen control, exposure in the adult Mongolian gerbil MG hormonal receptors expression, verifying alterations of hormone response later in life.

2. Material and Methods

2.1. Ethics statement and environment

The procedures described for these experiments were conducted according to the standards defined by the National Council of Animal Experiment Control (CONCEA, Brazil) and were approved by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP

(protocol number 113/2015). The animals were maintained in a BPA free isolator (Alesco, Monte Mor, SP, Brazil) placed in a rack with ventilation under controlled environment: room temperature of 23-26°C, 50% relative humidity, and light dark cycle of 12/12 hours. Fresh water and a commercial chow (Presença, Paulínia, SP, Brazil) were provided *ad libitum* to all animals during the experiment. Before the experiment began female Mongolian gerbils (*Meriones unguiculatus*) were kept with fertile males and pregnancy was confirmed with the presence of sperm cells in the vaginal smears – pregnancy day 1. The offspring obtained from treated pregnant/lactating animals were the focus of this experiment; they were maintained under the same environmental conditions until euthanasia was performed.

2.2. Animals and experimental design

The pregnant gerbils were divided in four groups (n=5). The first group was kept as intact control (G1) and did not receive any treatment. The exposed groups were set as follows: (G2) only corn oil (vehicle control; Mazola, Mairinque, SP, Brazil) daily; (G3) 35 µg/kg 17β estradiol (E8515, Sigma Aldrich, St. Louis, USA) (Pinto et al., 2008) diluted in 0,1 ml corn oil 3 times/week; and (G4) 50 µg/kg BPA (239658, Sigma Aldrich, St. Louis, USA) (Vandenberg et al., 2012) diluted in 0,1 ml corn oil daily. The treatments were performed from the 8th gestational day until the end of lactation (46 days of treatment); the treatment was suspended only on the parturition day. Gavage was performed always during the morning (8-10 am).

After weaning one female from each offspring (n=5) was randomly chosen and used for future analysis; other individuals obtained in the same offspring were used in other experiments. The selected females were maintained under the previously described conditions until reaching 180 days old. Then, all them were euthanized by stunning induced

with CO₂ followed by decapitation. The euthanasia procedures were performed during the morning with the animals always in estrous phase of the cycle, indicated by vaginal smears.

2.3. Biometrical analysis

During the procedures of euthanasia the body weight was evaluated before decapitation. After dissection the weights of liver and ovaries + uterus complex were obtained; the relative weight of these organs was calculated as a proportion, dividing it by the body weight. The organs were still evaluated for possible presence of macroscopic lesions.

2.4. Histological processing and cytochemical staining

For morphological and immunohistochemical (IHC) analysis the MGs were collected. The right abdominal gland was removed carefully and immediately fixed in paraformaldehyde 4% for 24 hours. Then, samples were transferred to alcohol 70% and histologically processed under a semi enclosed system (TP1020, Leica Biosystems, Buffalo Grove, USA). The paraffin blocks were sliced (4 µm thick) and cuts were put in glass slides. Cytochemical staining was performed with hematoxylin and eosin (HE) for a general morphological evaluation of the mammary glandular tissue and choice of the ideal slices to be evaluated by IHC evaluation.

2.5. Immunohistochemistry

The primary antibodies used were anti-ER α (ER α , rabbit polyclonal, IgG, MC-20, sc-542, Santa Cruz Biotechnology, Dallas, TX, USA); anti-ER β (ER β , mouse polyclonal, IgG2a, B-1, sc-390243, Santa Cruz Biotechnology, Dallas, TX, USA); anti-PGR (Alpha PR6, mouse monoclonal, IgG2a, GTX22765; GeneTex, Irvine, CA, USA), and anti-PRL-R (Anti-Prolactin receptor U5, mouse monoclonal ab2772, , Abcam, Cambridge, UK). Details on solutions and protocols adopted are described in Supplementary table. In summary, antigen retrieval was performed, endogen peroxidases were blocked by immersing the slides in 3% H₂O₂ at room temperature, and nonspecific proteins were blocked. The sections were incubated overnight

at 4°C with primary antibodies then secondary antibodies were applied for 1h at room temperature. Positive staining was detected by using 3-30'-diaminobenzidine tetrahydrochloride (DAB) chromogen (Novolink MaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstained with Mayer's hematoxylin.

Double IHC was also performed to check the concurrent expression of connexin 43 (Cx43) and ER α in cells of different MG compartments between the groups. For this protocol, slides were subjected to antigen retrieval in citrate buffer 0,1M (75 min at 98°C), endogenous peroxidase blockage in 0.3% H₂O₂ (30 min at RT), endogen biotin blockage (Avidin/Biotin blocking system, SP-2001, Vector Laboratories, Burlingame, CA, USA), inhibition of unspecific proteins with protein concentrate stock solution (Mouse on Mouse basic KIT, BMK-2202, Vector Laboratories, Burlingame, CA, USA) and incubation with first primary antibody (1:100, mouse anti-connexin 43, CXN6, sc-59949, Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. A secondary anti-mouse biotinylated antibody (rabbit anti-mouse E0464, Dako, Santa Clara, CA, USA) was used. The slides were then incubated with DAB for staining of Cx43. Immediately after, a second inhibition of unspecific proteins was performed, then the protocol was repeated: ER α antibody (1:250 rabbit monoclonal, NB300-560, Novus Biologicals, Centennial, CO, USA) was used for incubation overnight at 4°C, secondary antibody coupled to alkaline phosphatase (1:100 goat anti-rabbit IgG H&L, ab97048, Abcam, Cambridge, UK) was applied for 60 minutes at RT and the revelation was performed with fast red (TR/Naphthol AS-MX Tablets, F4648, Sigma Aldrich, St. Louis, MO, USA).

2.6. Quantification of cell expression and intracellular localization of hormonal receptors

For ER α , ER β and PGR, 30 random fields per group (n=5) were digitalized (400x magnification) using a BX61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120 Virtual Microscope Slide Scanning System (VS120-S5) from the same

company. The relative frequency of cells with positive nuclei was calculated in the MG secretory epithelium. The percentage was calculated by dividing the number of positive nuclei by the total number of cells counted. The results were expressed as percentages.

2.7. Protein expression in mammary gland samples (western blotting)

The left abdominal MG was removed and frozen at -80°C. Its protein content was extracted with a lysis buffer according to the protocol described by Li et al (2010). In summary, samples were smashed and left for extraction (CellLytic MT cell lysis reagent, C3228, Sigma Aldrich St. Louis, MO, USA) at 4°C during 1h under agitation. The protease activity was blocked by adding an inhibitor (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, USA). Then the samples were centrifuged for 20 minutes at 14000 rpm, 4°C. For quantification of protein concentration, a BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23227, Thermo Fischer Scientific, Rockford, IL, USA) was used and the absorbance was read in a microplate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany). The extracts were stored at -80°C until analysis were performed.

ER α , ER β and PGR were quantified in MG samples by Western blotting. Total protein amount of 15 μ g was placed in each well in the SDS page gel and subjected to electrophoresis (90 V for 120 minutes). The bands were transferred (100 V for 1 hour) to a nitrocellulose membrane (Amersham Protram, 10600003, GE Healthcare, Darmstadt, Germany). Blots were blocked using skimmed milk 5% in TBS + 0,1% tween (TBSt) for 1 hour at room temperature. Overnight incubation under agitation with anti- ER α , ER β , PGR (previously described) and GAPDH (mouse monoclonal MB374 Millipore, 1:10000), used as a positive control, diluted in skimmed milk 1% in TBSt was performed. Then, the membranes were washed in TBSt and incubated at room temperature for 1 hour under agitation with horseradish peroxidase conjugated secondary antibodies (1:10000 rabbit anti-mouse

ab6728, Abcam, Cambridge, UK, or 1:10000 goat anti-rabbit 115-035-144, Jackson ImmunoResearch, Jannersville, PA, USA). After washing the membranes antibody detection was revealed with ECL Substrate Pierce (32109, ThermoFischer, Rockford, IL, USA) and a Super Signal (34094, ThermoFischer, Rockford, IL, USA) reagents. The membranes were revealed in a imaging system machine (ChemiDoc MP, BioRad, Hercules, CA, USA) and the band densities were analyzed in a densitometry program – Image J (version 1.52a, Wayne Rasband, NIH, USA).

2.8. Hormonal analysis

Blood was collected from the neck immediately after decapitation. Samples were centrifuged (1200 g, 20 min) and serum was stored at -80°C for later hormonal analysis. Estradiol and progesterone were quantified in duplicate by capture/sandwich ELISA using specific commercial kits, according to the instructions of the manufacturer (estradiol EIA Kit, 582251, Cayman Chemical Company, Ann Arbor, MI, USA and progesterone Accu-Bind ELISA Microwells, 4825-300, Monobind Inc., Lake Forest, CA, USA). The readings were performed in a SpectraMax Plus 384 microplate reader (Molecular Devices, CA, USA).

2.9. Statistical analysis

Statistical analyses were performed by GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). First, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was adopted. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Influence of disruptors on biometric parameters

The administration of corn oil to mothers during gestation and lactation did not interfere in the weight of body, ovary + uterus complex nor liver of daughters after 6 months age (Table 1).

3.2. Influence of disruptors on hormone receptors expression and localization (immunohistochemistry)

The percentages of cells with positive nuclear staining for ER α increased in the G3 group ($34,7 \pm 3,4$) in comparison to the G1 group ($21,6 \pm 2,8$); the values found for G2 and G4 did not show any difference in comparison to other groups (Figure 1B). The percentages of ER β positive cells, however, did not show any differences between the groups (Figure 1D). The incidence of PGR positive cells was higher in G4 ($21,4 \pm 3,1$) in comparison to G1 ($8,2 \pm 0,5$).

The oscillating pattern of PRL-R expression did not allow the standard quantification of this protein. Thus, an evaluation of its expression pattern in both epithelial and stromal compartments between the groups was made. The samples from G1 showed PRL-R expression in the cytoplasm of fibroblasts in a very specific pattern: intense staining throughout the cytoplasm (Figure 2A-C). In this group, the secretory structures only showed positive staining in the fibroblasts (Figure 2A), being rare in epithelial cells. The stroma surrounding epithelial structures showed evident staining (Figure 2B). In G2 most the samples showed signs of hyperplasia, in which usually the epithelial cells were positive for PRL-R; the epithelial staining, however showed a different pattern in comparison to fibroblasts, being diffuse and badly delimited (Figure 2E,F). Both G3 and G4 groups showed positive epithelial cells with the same pattern seen in G2: G3 (Figure 2G), however, apparently showed less areas of positive epithelium in comparison to G4 (Figure 2H). Despite adipose tissue staining was present in all groups, it was clearly less frequent in G3 and G4.

3.3. Influence of disruptors on co-localization of ER α and connexin 43

Illustrative pictures of double IHC reaction for ER α and Cx43 are shown in Figure 3. It was observed that Cx43 was constantly present in cells negative for ER- α . The pattern of expression of Cx43 was always cytoplasmic, present only in the periductal region; ER- α was predominantly nuclear, despite some rare staining was present in the cytoplasm of some cells (Figure 3B'). Despite no quantification was performed, higher availability of Cx43 cells in the treated groups, especially in the BPA group, was present.

3.4. Influence of disruptors on protein expression (western blotting)

The quantifications of estrogen and progesterone receptor protein expression are shown in Figure 4. For ER α a similar pattern as that observed in the IHC quantification was present: an increase from G1 to G3 and a lower concentration in G4. Despite this similarity, the relative concentration in G4 was considerably lower in comparison to G2 and G3. ER β also showed lower concentration in G4 that differed statistically from G3. PGR expression, on the other hand, did not show differences between the groups.

3.5. Influence on serum estradiol and progesterone

The mean levels of serum estradiol were higher in intact control group ($328,8 \pm 92,45$ pg/ml) in comparison to vehicle control ($126,7 \pm 18,02$ pg/ml), 17 β estradiol ($57,06 \pm 11,16$ pg/ml) and BPA ($38,06 \pm 12,89$ pg/ml). However, no difference were found between G1 ($4,37 \pm 1,37$ ng/ml), G2 ($7,41 \pm 1,58$ ng/ml), G3 ($3,86 \pm 1,01$ ng/ml) and G4 ($5,17 \pm 1,24$ ng/ml) (Figure 5).

4. Discussion

The experiments performed in the present work assessed the protein expression of some hormone receptors. In terms of morphology evaluation, we found out that the number of cells expressing ER α was higher in the 17 β estradiol group in comparison to control and that the treatment with BPA increased the presence of PGR. The western blotting showed

differences between the BPA and the 17 β estradiol groups, not evidenced in the IHC analysis. Still, different patterns of PRL-R and Cx43 expression were described among groups. Regarding the serum concentrations of estradiol, there were significant decreases in all groups in comparison to intact control. In summary, the findings described above showed that perinatal exposure to xenoestrogen BPA and to 17 β estradiol is able to cause molecular alterations in the MG and fluctuations in the serum levels of estrogen, suggesting considerable long-term repercussions as observed in other experiments (Acevedo et al., 2018; Mandrup et al., 2013).

Despite the known obesogenic effect of some endocrine disruptors (Grün and Blumberg, 2006), the body weight showed no significant changes between the groups; in the same way, ovary + uterus and liver weights did not change. Contrarily, at 21 weeks age, rats showed higher body weight after perinatal BPA treatment (Xia et al., 2014). Despite that, the authors did not observe any difference in absolute neither in relative liver weights between treated and control animals, as seen in our results. Increases in liver weight due to hepatotoxic injuries may be caused by inflammation related to adduct formation, leading to hepatic scar depositions in long term or proliferative lesions (Zachary and McGavin, 2013); in addition to that the necessity of BPA metabolism during fetal life may cause adult metabolic diseases as observed in other experiments with rodents (Le Magueresse-Battistoni et al., 2018). The results shown by our evaluations suggest that the treatment adopted was not sufficient to cause such systemic alterations in Mongolian gerbils, not even in terms of female reproductive tract, as seen by similar weights of ovary + uterus complexes.

Contrarily, steroid hormone receptors showed responses to the treatments. Their expression in mammary tumors is usually a biological marker for prognosis prediction and treatment decisions. More specifically, ER α and PGR have been linked to breast cancer risk in humans

(Burns and Korach, 2012; Khan et al., 1998). However, the relation of both receptors expression was not observed in a study performed a decade ago, where the authors found out that among postmenopausal women, breast cancer risk was inversely associated to expression of ER α and PGR (Lagiou et al., 2009).

In Mongolian gerbils, western blotting analysis of hormone receptors performed so far are limited to some organs, such as testes (Negrin et al., 2018) uterus and stomach (Saqui-Salces et al., 2008); this is the first description of ER α , ER β and PGR protein bands detection in MG of this rodent species. Bands of 68 kDa were detected for ER α , 56 kDa for ER β and 120 kDa for PGR, suggesting the exclusive detection of PGR-B isoform (reference: www.genetex.com). With the exception of ER β , the other receptors have been described in gerbils by Saqui-Salces et al, who could not detect ER β bands and found 85 kDa PGR bands, indicating PGR-A isoform. These differences may have been due to the use of different antibodies and evaluation of different tissues; still, this may consist in a specificity of Mongolian gerbils.

In the present experiment IHC analysis showed lower expression of ER α in the intact control in comparison to the 17 β estradiol treated group; however, no difference was present when comparing it to BPA exposed group. Western blotting, in contrast, was able to evidence that the slight reduction of ER α expression in G4 shown by IHC is significant when the whole tissue is evaluated. Since our IHC quantification was performed exclusively in the epithelial compartment, it is possible to assume that the ER α expression in other mammary components is significantly higher in G2 and G3 groups. Indeed, positive cells for ERs and PGR were present in the stromal compartment. For PGR, no different results were found, showing that this receptor does not vary between the groups as an effect of BPA nor 17 β estradiol. ER β , however showed lower expression in BPA treated animals. In terms of human

disease no correlation was demonstrated for ER β and breast cancer malignity (Borgquist et al., 2008). Still, the expression pattern of this receptor tends to be higher in the involution phases, as described before for Mongolians gerbils (Leonel et al., 2017), suggesting that this receptor is not associated to MG proliferation. Indeed, it was previously stated that ER β serves to facilitate ER α return to the nucleus, restoring its responsiveness to estrogens (Cheng et al., 2004), not being related to proliferative malignancy of the MG epithelium.

Regarding the pattern of expression, according to the literature, in humans ER α and PGR tend to be more localized in epithelial cells, whereas ER β tends to be more widespread (Burns and Korach, 2012; Li et al., 2010). Our analysis by IHC and blots has shown similar fluctuation patterns for both ER α and ER β between the groups. However, the statistical difference observed in ER β blots between G4 and G3 suggests that this receptor is more expressed in the non-epithelial compartment of the gland in G3. According to Paech et al (1997), the presence of estrogen makes ER α an activator and ER β an inhibitor of transcription (Paech et al., 1997). However, other studies suggest that ER β plays an independent regulator role with distinct cellular functions (Saji et al., 2000), including a protective role since this receptor can down-regulate ER α transcription capacity (Paech et al., 1997). With that, since ER β is normally more abundantly expressed in rodent MG (Saji et al., 2000), its reduction in G4 may indicate a malignant effect caused by perinatal exposure to BPA.

Contrarily to estrogen and progesterone receptors, PRL-R showed different patterns of expression including nuclear and cytoplasmic reactivity. Thus, we decided to make a description of its cell localization and relate it to what was previously described in the literature for other species, since no information regarding Mongolian gerbils was available. The presence of PRL-R in epithelial cells is increased during pregnancy and lactation in the

rat model, suggesting its necessity during proliferation and milk production MG phases (Camarillo et al., 2001). In summary that work states that stromal expression of PRL-R does not change among different developmental stages; epithelial expression, however, considerably increases in pregnancy and lactation, leading one to conclude that prolactin action shifts to epithelium in fully differentiated MG cells. The constant presence of PRL-R in the fat pad may be explained by its regulation of lipoprotein lipase (Hang and Rillema, 1997), an important enzyme for fatty acid metabolism. Other experiments, however, must be performed towards this point of interest to answer to this question.

The Cx43 MG malignancy marker, also known as gap junction protein alpha 1, is normally localized in cell junctions but also in vesicles in the cytoplasm and nucleoplasm (Thul et al., 2017). Despite this protein function is normally a component of gap junctions; we could not find it in the epithelial cells, as expected. The idea that Cx43 is linked to MG malignancy (Plante et al., 2011) led us to evaluate the relation between its expression and that of ER α , a marker for cells that respond to estradiol and tend to proliferate. However, Cx43 was only present in cells not expressing ER α , normally present in epithelial cells. In fact, the family of gap junction proteins, in addition to make intercellular communication, is involved with cell proliferation, cell differentiation and cell homeostasis phenomenon (Hervé et al., 2007). Previous experiments describe the expression of Cx43 in the myoepithelial cells (Pozzi et al., 1995; Yamanaka et al., 1997), suggesting that they were interconnected by gap junctions formed by Cx43. According to Yamanaka et al (1997), this expression increases right after delivery in myoepithelial cells, suggesting an important role of Cx43 in the lactating process (Plum et al., 2000). The expression of Cx43 in the Mongolian gerbil MG was present as punctate plaques, as described before (Stewart et al., 2013). The presence of Cx43 in the cytoplasm suggests a preparation of cells that will need more contraction abilities, such as its

pattern of expression in the endometrium during pregnancy, which increases in preparation to birth but is concentrated in the cytoplasm; at birth time, it migrates to the plasma membrane, where it will exert its function (Hendrix et al., 1992). Thus, an apparent increase of Cx43 positive cells in G4 may be related to a protective response of the gland due to a possible risk environment, since Cx43 is frequently present in stroma of invasive carcinomas (Jamieson et al., 1998; McLachlan et al., 2007).

Despite some experiments report the increase in serum estradiol after BPA treatment in mice (Xi et al., 2011) and in rats (Fernández et al., 2010) we found a considerable decrease on this parameter. Xi et al assume that the effects of perinatal BPA administration cause alterations on gonad steroidogenesis; however, these authors evaluated pups, while Fernández et al analyzed the serum from 4-5 month old rats. These authors assume that the high estrogen levels found were due to the presence of ovarian cysts, which according to Crain et al. (2008) are the cause of high serum levels of BPA due to a reduction of BPA clearance (Diamanti-Kandarakis et al., 2009). The decrease in serum estradiol levels after perinatal treatment with BPA and ethinyl estradiol has been observed in rats (Patel et al., 2017), similarly as described in the present experiment. A presumable explanation for the increase of estradiol levels in that experiment is the route of exposure, subcutaneous, which may result in the absorption of higher levels of BPA in comparison to the oral route. It is important to state that in our experiment the euthanasia was performed always in the same phase of estrous cycle, avoiding changings due to the natural cyclic variation of hormonal production.

In order to answer to the main question of this work, one should speculate the results to evaluate similarities and differences between the mechanisms of action of BPA and 17 β estradiol. The endogen estradiol mechanism of action is well known and is based exclusively

on receptor binding. BPA, however may have its action after binding to different receptors, such as peroxisome proliferator-activated receptor γ (Delfosse et al., 2014), to non canonical estrogen receptors not localized in the nucleus (Marino et al., 2012) or even inhibit the androgen receptors action (Delfosse et al., 2014). Thus, even though we could not specify the actual mechanism of action, the effects of BPA and 17β estradiol perinatal exposure in the adult female MG is distinct and peculiar for this species. Still, the decrease in serum estrogen may have led to a modulation or disruption of the estrogenic pathway in adult female gerbils exposed during neonatal life.

In conclusion, our results indicate that perinatal exposure to BPA and 17β estradiol may alter the expression of ERs allowing hormonal disruption in adulthood; the decrease in serum estradiol levels supports this conclusion. It also affects the levels of PGR epithelial expression and the localization pattern of PRL-R, affecting also the responses to progesterone and prolactin in the MG of female gerbils.

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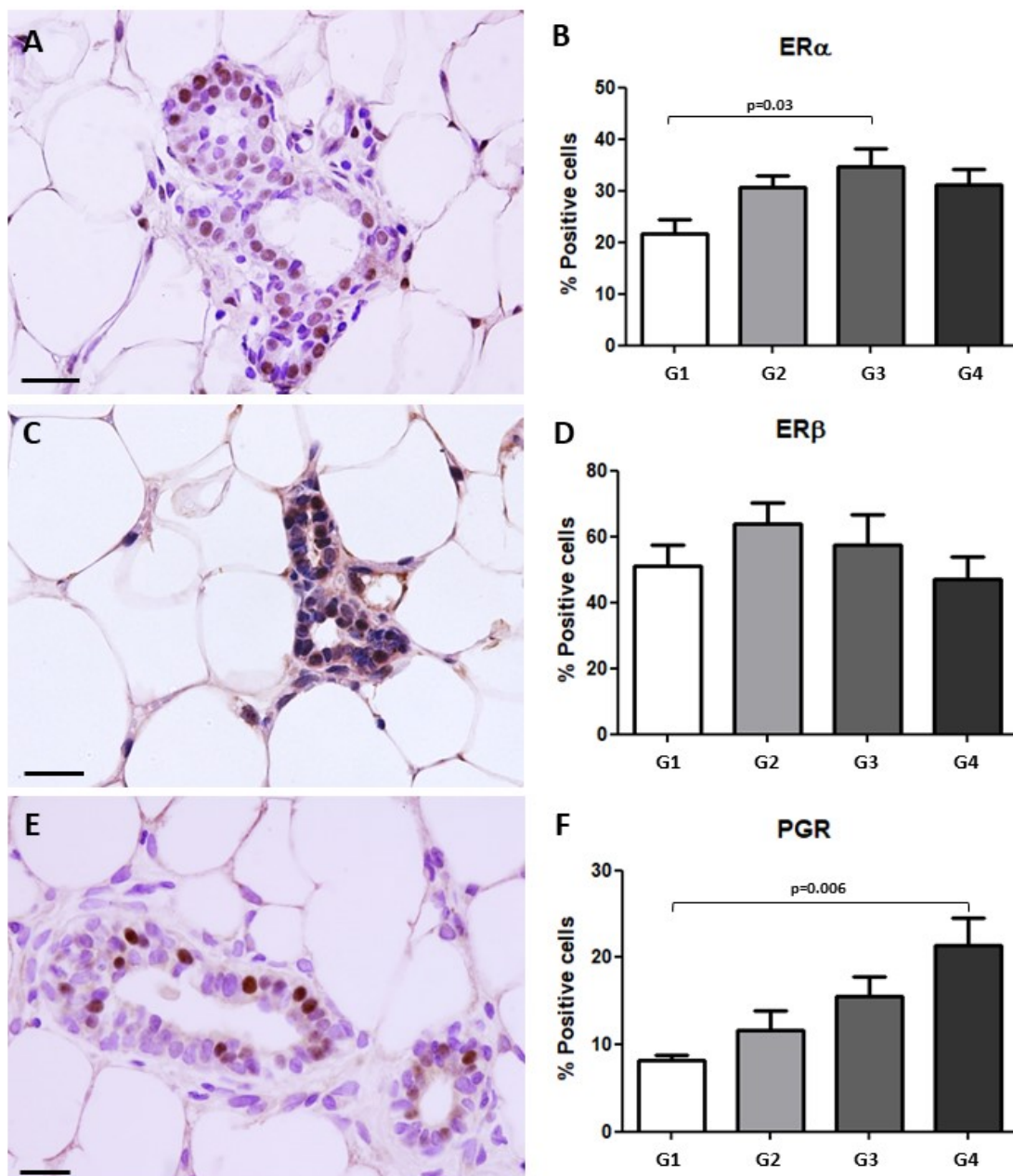


Figure 1. Illustrative photomicrographs of nuclear receptors ERα (A, G3), ERβ (C, G2) and PGR (E, G4) immunostaining (counterstaining with Meyer's hematoxylin) and percentages of positive cells. Values (mean ± SEM) were considered significant when $p < 0.05$ (ANOVA test followed by Tukey's test in B and D, Kruskal Wallis test followed by Dunn's test in F). Bars: 25 μm .

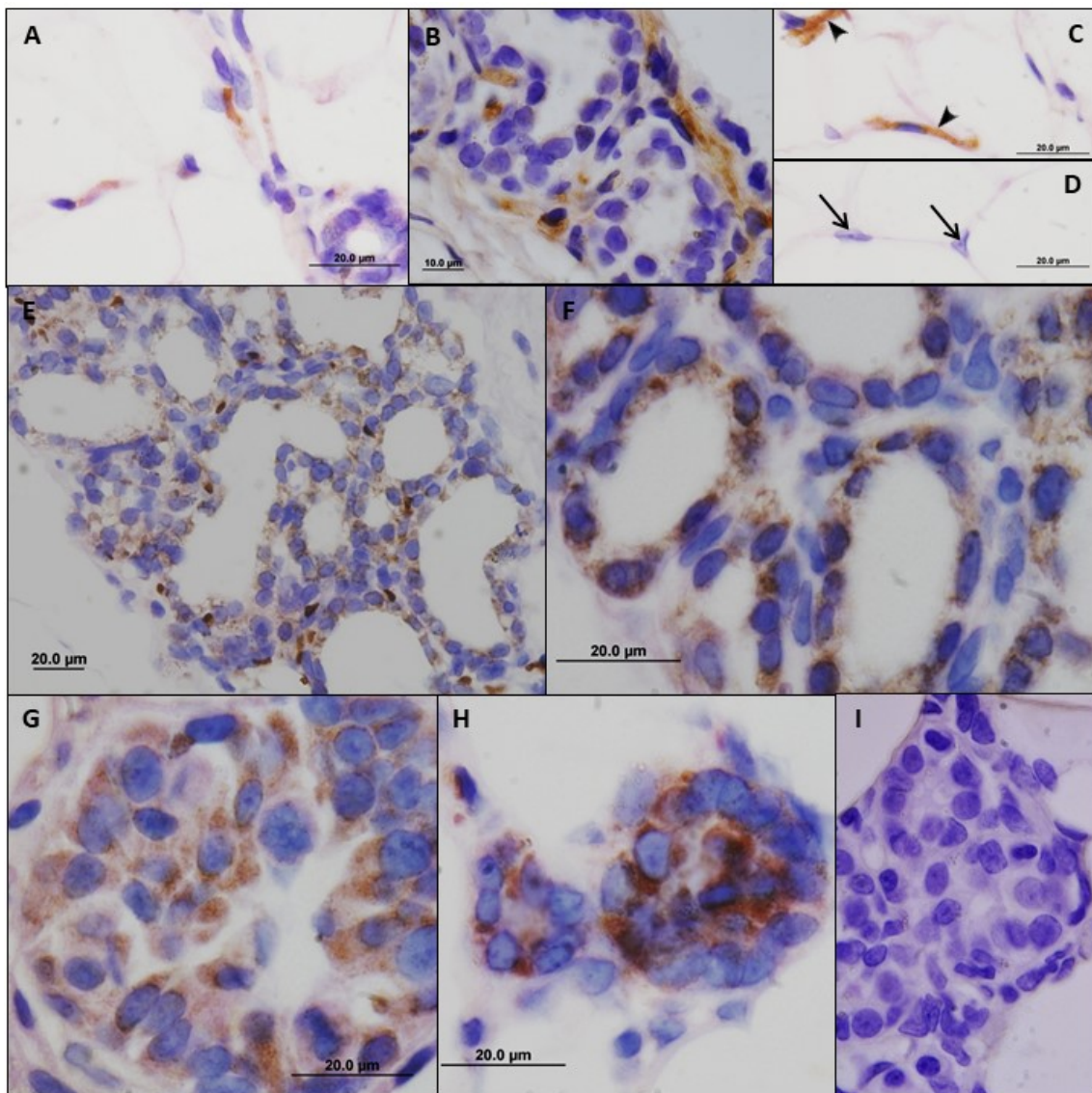


Figure 2. PRL-R staining pattern in the mammary gland tissue (counterstaining with Meyer's hematoxylin). G1 showed a constant pattern of staining among adipose tissue (A), surrounding the epithelial structures (B), specifically in fibroblasts (arrowheads) (C) but not in adipocytes (arrows) (D); in this group the staining of epithelial cells was rare. G2 showed high frequency of hyperplastic tissue, which usually presented positive staining of epithelium (E,F). G3 (G) and G4 (H) showed epithelial cells staining as well. Negative control is shown in (I). Bars: 10 μm in B and 20 μm in A, C-H.

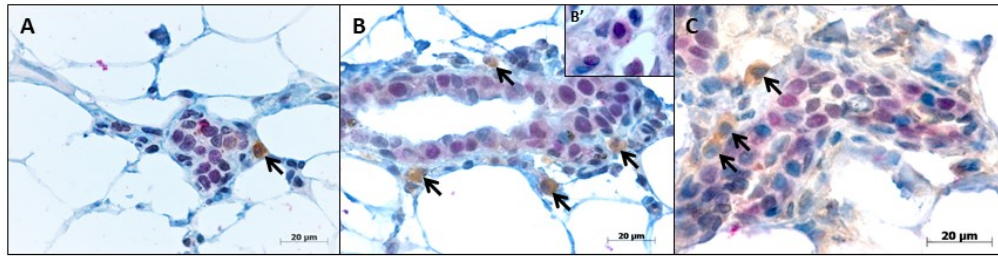


Figure 3. Illustrative photomicrographs from mammary gland subjected to double immunohistochemistry with Cx43 (brown) and ERα (pink) in G1 (A), G3 (B) and G4 (C). There was no protein expression overlapping in the cells. Some cytoplasmic staining of ERα was observed (B'). Bars: 20 μm.

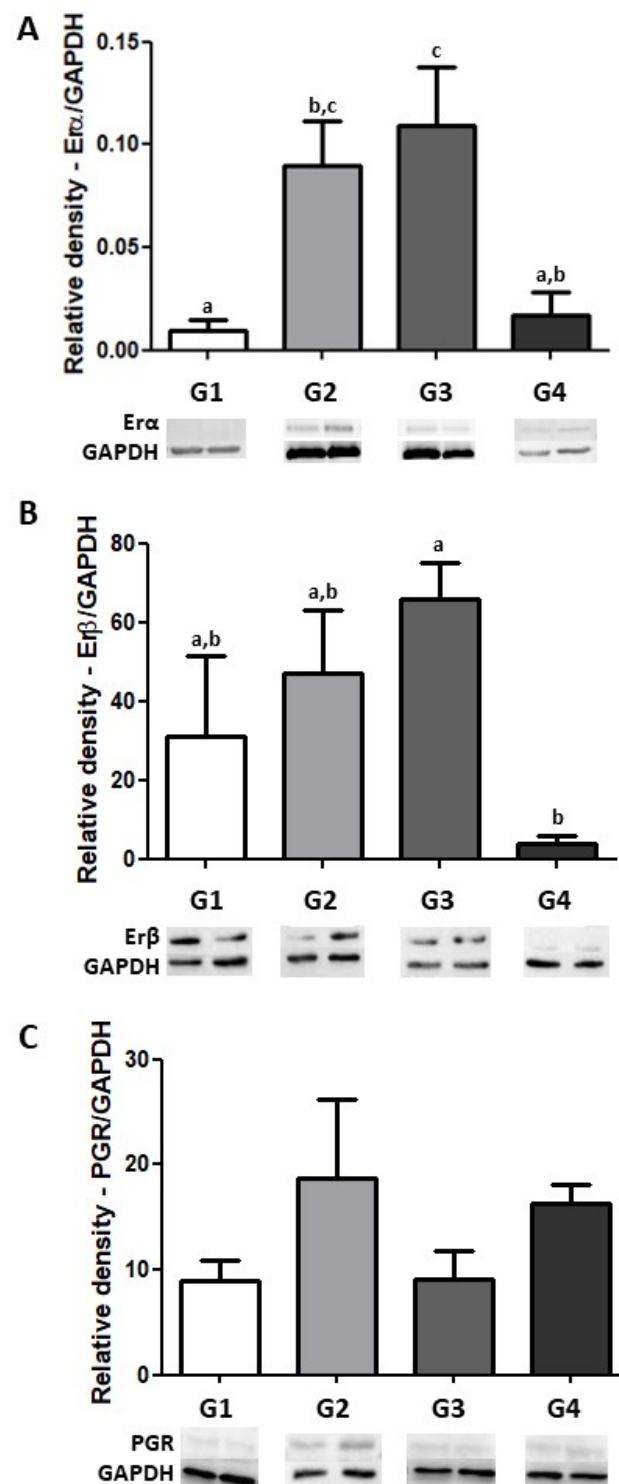


Figure 4. Relative density of $ER\alpha$ (A), $ER\beta$ (B) and PR (C) normalized by GAPDH here used as positive control. n=4 animals per group. Values are expressed as mean $\pm$ SEM, $p < 0.05$ (ANOVA followed by Tukey's test).

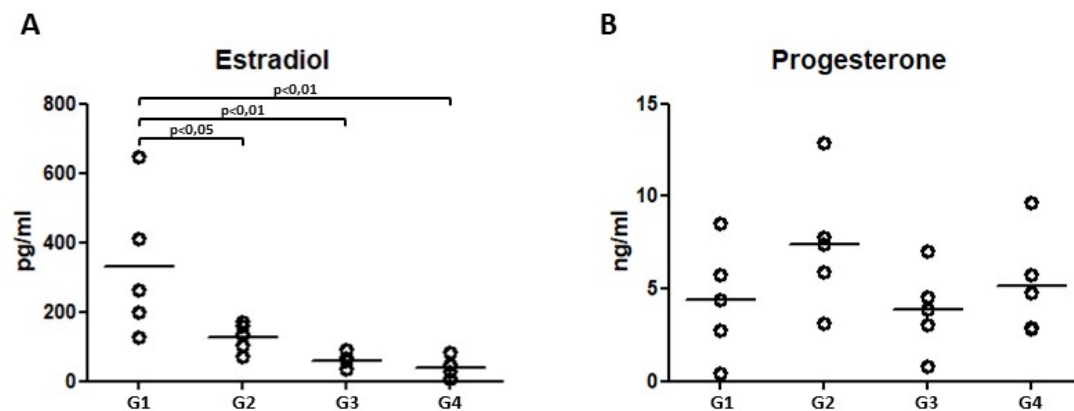


Figure 5. Serum hormonal profile of animals from intact control (G1), vehicle control (G2), 17- β estradiol (G3) and BPA (G4) groups. The estradiol (A) and progesterone (B) dosages were performed in 5 animals per group. Individual values (circles) and mean values (lines) are shown. Values were considered significant when $p < 0.05$ (ANOVA test followed by Tukey's test).

Table 1. Biometric data (g) of animals from intact control (G1), vehicle control (G2), 17 β estradiol (G3) and BPA (G4) groups.

Biometric parameters	Experimental groups			
	G1	G2	G3	G4
Body weight^a	71.6 $\pm$ 4.8	70.6 $\pm$ 1.5	68.5 $\pm$ 2.3	72.4 $\pm$ 2.4
Ovary+Uterus^b	0.26 $\pm$ 0.04	0.27 $\pm$ 0.03	0.24 $\pm$ 0.04	0.28 $\pm$ 0.03
Ovary+Uterus (relative weight x10⁻³)^b	3.63 $\pm$ 0.36	3.83 $\pm$ 0.52	3.51 $\pm$ 0.59	3.88 $\pm$ 0.44
Liver^a	2.68 $\pm$ 0.17	2.74 $\pm$ 0.15	2.65 $\pm$ 0.11	3.01 $\pm$ 0.13
Liver (relative weight x10⁻²)^a	3.78 $\pm$ 0.25	3.86 $\pm$ 0.15	3.88 $\pm$ 0.12	4.12 $\pm$ 0.11

Values expressed as mean $\pm$ SEM. ^aANOVA followed by Tukey's test, ^bKruskall Wallis followed by Dunn's test

Supplementary table. Immunohistochemistry protocols adopted for each antibody.

Anticorpo	Antigen retrieval	Endogenous peroxidase blockage	Inhibition of unspecific proteins	Antibody concentration	Secondary antibody
ERα	Citrate buffer 10mM pH 6.0, 15 min water bath at 96°C	H ₂ O ₂ in methanol (10%), 2 baths of 15 min, RT	BSA 1% in TBS, 60 min, RT	1:50	Secondary antibody (1:200), 45 min followed by Avidin and Biotin, 45 min
ERβ	Tris + EDTA, 3 cycles of 7 min in microwave (low power)	H ₂ O ₂ in methanol (10%), 20 min, RT	Skimmed milk 5%, 30 min, RT	1:50	Secondary antibody (1:200), 45 min followed by Avidin and Biotin, 60 min
PGR	Tris+EDTA, 30 min water bath at 96°C	H ₂ O ₂ in methanol (10%), 20 min, RT	Skimmed milk 5%, RT, 20 min	1:50	Kit Polymer, Novolink, Leica, RT, 60 min
PRL-R	Tris+EDTA, 30 min water bath at 96°C	H ₂ O ₂ in methanol (10%), 20 min, RT	Skimmed milk 5%, RT, 20 min	1:100	Kit Polymer, Novolink, Leica, RT 60 min

BSA: bovine serum albumin, RT: room temperature

CONCLUSÃO

Nos trabalhos realizados pudemos observar que a exposição perinatal ao BPA e ao 17 β estradiol, de fato podem repercurir na fisiologia mamária em longo prazo, possibilitando a ocorrência de alterações tanto na morfologia quanto na expressão de proteínas relevantes para a resposta hormonal.

A caracterização morfológica da GM normal de gerbilos da Mongólia descrita no primeiro capítulo foi essencial para que alterações patológicas pudessem ser avaliadas posteriormente, uma vez que tal caracterização nesta espécie não havia ainda sido descrita.

Os resultados do segundo capítulo levam a concluir que BPA administrado durante o desenvolvimento perinatal afeta negativamente a arquitetura glandular em animais adultos, podendo predispor estes animais a lesões neoplásicas. Pôde-se concluir também que o óleo de milho usado como veículo aumenta o risco de desenvolvimento tumoral na GM, já que os índices de expressão de EZH2 aumentaram neste grupo.

No terceiro capítulo observou-se que a exposição perinatal ao BPA e a 17 β estradiol leva à desregulação hormonal durante a idade adulta, visto que houve alterações nas concentrações hormonais séricas bem como mudanças na expressão de receptores hormonais nas células mamárias.

Assim, os trabalhos desenvolvidos levam à conclusão geral de que, apesar dos níveis permitidos de utilização de BPA na manufatura de plásticos e resinas serem reduzidos, estes ainda podem repercutir negativamente na GM quando presentes durante os primeiros estágios de desenvolvimento dos indivíduos. As alterações morfológicas observadas, apesar de não caracterizarem alterações malignas propriamente ditas, indicam presença de pré malignidade, revelando a possibilidade de posterior desenvolvimento tumoral naqueles indivíduos expostos em comparação com indivíduos controle. Da mesma forma, as alterações em níveis hormonal e de receptores predispõem o indivíduo a posterior desenvolvimento neoplásico. Deve-se ressaltar que, apesar de limites no uso de BPA serem impostos, este componente reduz os custos na produção de materiais manufaturados. Isto pode significar, muitas vezes, um maior risco de exposição presente no dia a dia de populações menos favorecidas economicamente, que muitas vezes não têm acesso a materiais fiscalizados e produzidos de acordo com as normas específicas. Isto

torna pesquisas com este desregulador endócrino cada vez mais importantes com relação à conscientização e melhor fiscalização na produção destes consumíveis.

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ANEXO

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Stepped vitrification technique for human ovarian tissue cryopreservation

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ABSTRACT

Purpose: The advantage of stepped vitrification (SV) is promoting vitrification, thus avoiding ice crystal nucleation, while decreasing the toxic effects of high cryoprotectant concentrations. We aimed to test this method for human ovarian tissue cryopreservation.

Methods: Ovarian cortex was taken from 7 fertile adult women. Samples were subjected to an SV protocol performed in an automatic freezer, which allowed sample transfer to ever higher concentrations of dimethyl sulfoxide (DMSO) as the temperature was reduced. Once the highest cryoprotectant concentration (50%) was achieved at -40°C , the samples were vitrified in vapors, plunged into liquid nitrogen, and stored until the warming procedure was initiated (also in a stepwise manner). The reason why follicle integrity was not maintained was investigated, so the next analysis evaluated the efficacy of DMSO perfusion by X-ray computed tomography, ice crystal formation by freeze-substitution, and cell toxicity by transmission electron microscopy.

Results: Histological evaluation of the vitrified-warmed tissue showed large numbers of degenerated follicles after 24 hours of in vitro culture. Although cryoprotectant perfusion was considered normal and no ice crystals were formed in the tissue, ultrastructural analysis detected typical signs of DMSO toxicity, such as mitochondria degeneration, alterations in chromatin condensation, cell vacuolization and extracellular matrix swelling, in both stromal and follicular cells.

Conclusions: The findings indicated that the method failed to preserve follicles due to the high concentrations of DMSO used. However adaptations can be made to avoid toxicity to follicles caused by elevated levels of cryoprotectants.

KEYWORDS: Dimethyl sulfoxide, toxicity, ovarian follicle, ultrastructure, stepped vitrification, cell membrane.

1. Introduction

These days, human fertility preservation should be considered a complement of cancer treatments (Turan and Oktay, 2014). Indeed, thanks to substantial improvements in treatment and prognosis, ovarian tissue cryopreservation is a technique that has been gaining ground (De Felice et al., 2018), not only to safeguard fertility, but also to maintain hormone levels in women subjected to gonadotoxic treatments or in the menopause (Woodtli et al., 2018). Results obtained so far are promising, with more than 130 babies born to date, and the technique is now on the verge of becoming a possible routine approach and no longer experimental (Gellert et al., 2018).

Ovarian tissue obtained from these patients is a very precious source of germ cells for fertility preservation, so assays to improve its manipulation have been constantly under development. Slow-freezing (SF) is the most common method used for human ovarian tissue cryopreservation today (Donnez et al., 2013), derived from a protocol described several decades ago for sheep ovarian tissue (Gosden et al., 1994).

The main challenge faced during freezing is formation of ice within the tissue, as reducing the temperature leads to ice crystal formation due to ice nucleation. This phenomenon is followed by crystal growth when the molecular arrangement of water is altered to a solid pattern (Wowk, 2010). When the temperature falls very fast, there is no time or energy for molecular rearrangement, so there is no ice crystal formation; the natural disorder of liquid molecules in tissue is maintained, mitigating any disturbance to the system. This forms the basis of the vitrification concept, as the absence of crystals prevents cell membrane damage normally caused by their mechanical activity. While vitrification protocols are already well established for oocytes (De Munck et al., 2015) and embryos (Murakami et al., 2014), tissue vitrification remains a challenge because of its variety in composition and size.

Vitrification procedures have been optimized by increasing the viscosity of the solution, which lowers the melting temperature (MT) of the sample and results in its dehydration. In normal conditions, if temperatures drop before the MT is reached, ice formation occurs. However if the liquid viscosity is increased, crystal formation is avoided (Kauzmann, 1948, Wowk, 2010). On the other hand, an increase in viscosity requires high concentrations of cryoprotective agents (CPAs) in order to extract water from the tissue, which may create other problems, namely toxic effects suffered by cells (Mazur et al., 1972).

To avoid this issue, samples can be subjected to a low temperature environment, with low rates of potential energy and cell metabolism. Consequently, molecules become less mobile and more stagnant, but remain disordered. Based on work first described by Farrant in 1965 (Farrant, 1965), a procedure known as liquidus tracking was developed by Pegg et al. to cryopreserve cartilage (Pegg et al., 2006), involving enhancement of CPA concentrations in samples while decreasing their temperature (Fahy et al., 1984). This equilibrium procedure prevents ice crystal formation (thanks to high concentrations of CPAs) and decreases cell susceptibility to toxic effects (thanks to low temperatures). The same technique has been tested in bovine ovarian tissue (Corral et al., 2018), yielding good rates of CPA perfusion and morphology maintenance. Nevertheless, species-specific responses may vary, especially in human tissue. The aim of this work was therefore to evaluate the effectiveness of a stepped vitrification (SV) protocol based on the liquidus tracking technique in terms of maintenance of the structural characteristics and morphology of human ovarian follicles.

2. Material and methods

2.1 Ethics and experimental design

Ovarian tissue was taken from 7 women (from 27 to 33 years of age) undergoing laparoscopic procedures for benign gynecological diseases after obtaining informed consent. Use of human ovarian tissue was approved by the Institutional Review Board of the Université Catholique de Louvain on November 28, 2016 (IRB reference 2012/23MAR/125, registration number B403201213872).

After removal, ovarian biopsies were immediately transported to the laboratory in Dulbecco's Modified Eagle Medium (DMEM / F12; Gibco, USA) in sterile and low temperature conditions (0°C). Once in the laboratory, the tissue was washed and cleaned of its medullary part, before being cut into of 5x5x1 mm pieces and subjected to the previously described vitrification procedure (Corral et al., 2018). Small fragments (1x1x1 mm) were used as fresh controls and immediately fixed for analysis by light and transmission electron microscopy (TEM). Short-term (24 hours) in vitro culture (IVC) of fresh ovarian tissue (1x1x1 mm³) was also performed for control purposes to ensure culture protocol effectiveness for cell morphology maintenance and activity. All mentioned protocols are described below. All chemicals used were purchased from Sigma-Aldrich, unless otherwise stated.

2.2 Vitrification procedure

A programmable controlled-rate freezer was used (Asymptote, General Electric Healthcare, VIA Freeze Research, Sovereign House, Cambridge, UK). It was equipped with an SV device designed to move samples to vials with different concentrations of dimethyl sulfoxide (DMSO).

Four 3 ml vials were filled with minimal essential medium (MEM + GlutaMAX; Gibco, USA) containing increasing concentrations of DMSO (10%, 20%, 40% and 50%), 8% human platelet lysate (Macopharma, France) and 2 IU/ml heparin (LEO Pharma, Denmark). The vials were then placed on the cooling plate of the freezer and remained open throughout the vitrification procedure, so tissue samples could move freely from one vial to another (Fig. 1). The entire procedure was performed inside a laminar flow hood under sterile conditions.

The samples were subjected to DMSO permeability in low temperatures, as demonstrated by the curve shown in Figure 1. During each bath, ovarian tissue was kept submerged in medium for different periods of time, and moved in a constant up-down motion inside a small plastic basket in order to improve CPA perfusion, promoting faster equilibration of the tissue. In short, the ovarian cortical tissue was subjected to a 10% v/v DMSO bath at 0°C. The samples were then transferred to ever higher concentrations of DMSO concurrently with a temperature drop in the machine (20% DMSO at -4°C, 40% DMSO at -8°C, 50% DMSO at -25°C). Finally, the temperature was reduced to -40°C and the samples were placed in cryovials containing pre-cooled (-40°C) 50% DMSO solution to proceed with vitrification. Temperature monitoring was carried out using a thermocouple data logger deposited inside a side vial containing 50% DMSO solution. The vials were kept in liquid nitrogen vapors (approximately 2 cm above the liquid nitrogen surface) inside a cryotank for 5 minutes and vitrified to -150°C. They were then submerged and stored in liquid nitrogen until warming.

2.3 Warming procedure

An adapted previously described slow-warming protocol (Pegg et al., 2006) was applied. Briefly, the cryovials were removed from the liquid nitrogen and placed in the pre-cooled programmable freezer (-40°C) for 30 minutes. Thereafter, the solution was replaced in order to remove the CPA from the samples. Lower DMSO concentrations and

higher temperatures were used, as shown in Figure 1. The samples were kept in a 40% DMSO solution at -20°C for 30 minutes, then in 30% DMSO for another 30 minutes. At 0°C, 20% and 0% DMSO solutions were utilized for 30 minutes each. Finally, three baths without DMSO lasting 5 minutes each were performed at room temperature (RT) (23°C). The ovarian tissue fragments were then divided into 1x1x1 mm pieces that were either subjected to 24 hours of IVC, as described below, or fixed for TEM evaluation.

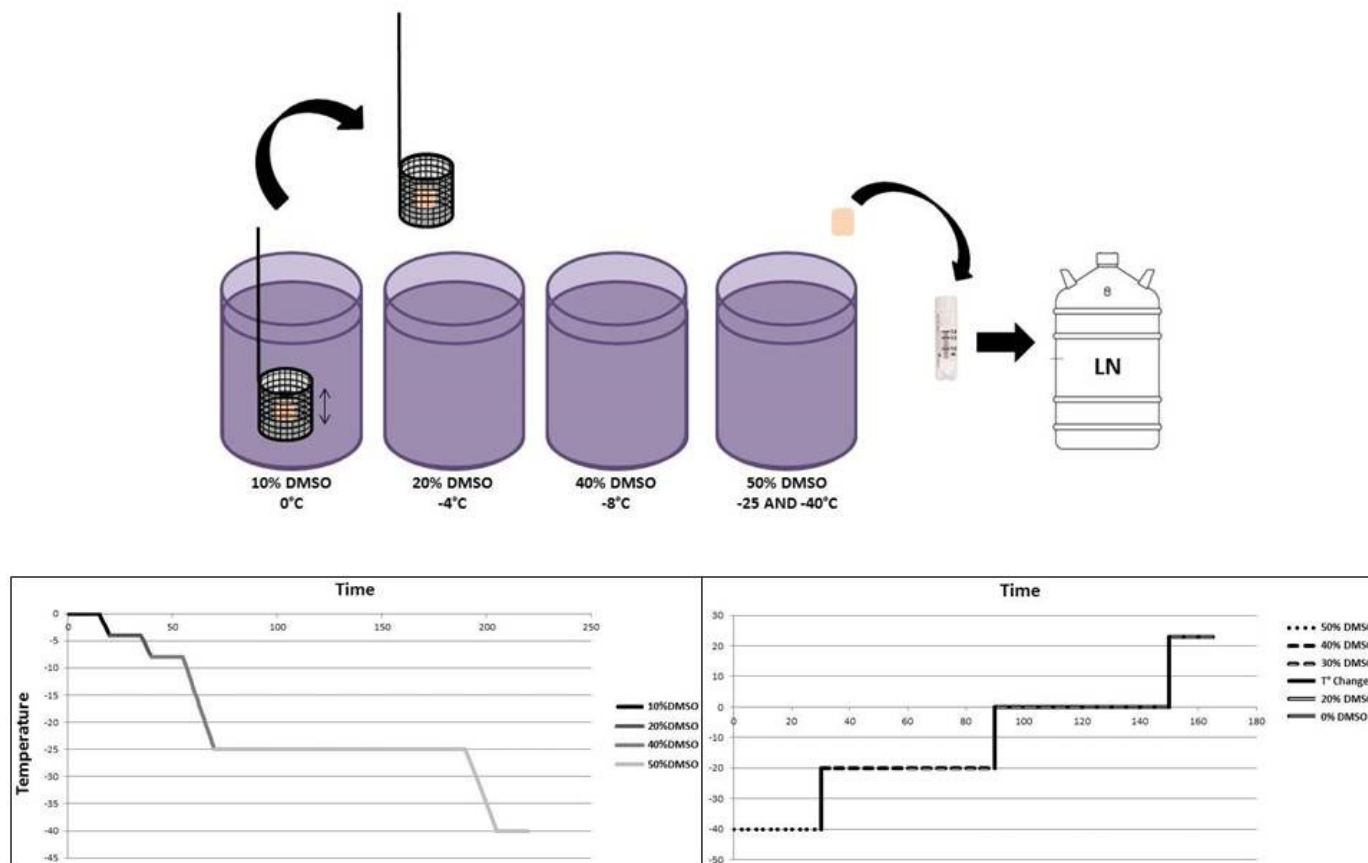


Fig 1 Equilibrium vitrification system and cooling and warming curves achieved in the programmable freezer with the stepped vitrification device. DMSO: dimethyl sulfoxide; LN: liquid nitrogen

2.4 24h in vitro culture

Fragments of fresh and warmed ovarian cortex were divided into 1x1x1 mm pieces and in vitro cultured in an attempt to restore the activity of follicles and other ovarian cells, and determine the impact of the cryopreservation procedure on their viability. The basic medium used was the same as that described for vitrification solution enriched with insulin, transferrin and selenium (ITS, Gibco, USA, 1%), pyruvic acid (2 mM), ascorbic acid (50 µg/ml) and antibiotics, namely amphotericin B, penicillin and streptomycin (Gibco, USA, 1%). The cortical pieces were kept inside a 5% carbon dioxide (CO₂) incubator (Thermo Fischer Scientific, USA) at 37°C. After 24 hours of culture, they were fixed in 4% paraformaldehyde and processed for histological analysis.

2.5 Histological analysis

After fixation in 4% paraformaldehyde, fresh and cultured samples underwent histological processing (dehydration and clarification) before being embedded in paraffin. The blocks were then subjected to 5- μ m-thick sectioning in a microtome and the slides were stained with hematoxylin and eosin for a general analysis of the tissue morphology and follicle counting.

Follicles were classified as morphologically normal when they had an intact spherical aspect, with uniform distribution of granulosa cells and a spherical oocyte showing no retractions or vacuoles attached to the surrounding stroma. In the absence of these characteristics, follicles were considered to be degenerated. Only those exhibiting a visible oocyte nucleus were counted.

2.6 X-ray computed tomography

To determine CPA perfusion and removal efficiency, samples from three different patients were analyzed by computed tomography (CT) in order to ascertain DMSO concentrations in ovarian tissue after vitrification. A NanoCT scanner (Bioscan, USA; Mediso, Hungary) was used for this purpose, as previously reported (Corral et al., 2015, Corral et al., 2018). The equipment was set up to maintain the samples at temperatures below -140°C during vitrified tissue analysis. Once the temperature was reached, the samples were transferred from liquid nitrogen to the insulating container and CT image acquisition was performed. Specimens were then subjected to the warming protocol and NanoCT images were acquired at RT. This investigation was conducted to evaluate the washes for DMSO removal by searching for any remaining CPA.

Acquisition parameters included a 106 mA current for a voltage of 65 kV, exposure time per projection of 500 ms, and 360 projections per rotation. Each image required 3 minutes for total acquisition and was reconstructed to a spatial resolution of 0.2 mm. For imaging, three different software programs were used, namely Nucline software for acquisition (Mediso, Hungary), IVS image processing software for reconstruction (Invicro, USA), and PMOD 3.7 for analysis (PMOD Technologies LLC, Switzerland). For image visualization, a PMOD cold scale was applied, where the lowest attenuation is represented by a dark blue color and the highest attenuation by intense red, passing through green, yellow and orange for intermediate attenuation values.

2.7 Freeze-substitution

The freeze-substitution technique enables visualization of possible ice formation in frozen or vitrified samples. Its main advantage is allowing preservation of tissue morphology as it is prior to warming, by fixing and processing fragments at low temperatures (Shiurba, 2001). To this end, our samples were removed from the liquid nitrogen and transferred to a temperature of -80°C. The medium was replaced with a solution containing 1% osmium tetroxide in methanol, which replaces water in tissues when changed over a series of 5 days. The samples were kept at -20°C for 24 hours, before being warmed to 4°C and left for a further 2 hours. The ovarian fragments were then dehydrated in a propylene oxide solution and infiltrated with epoxy resin. Sections measuring 2.5 μ m in thickness were obtained and stained with toluidine blue, before analysis by light microscopy.

2.8 Transmission electron microscopy

After fixation in Karnovsky's solution, the samples were processed as previously described (Borges et al., 2009). Ultrastructural organization was evaluated only in fresh controls and tissue obtained from 3 patients soon after

warming, which showed higher follicle density. Post-fixation was achieved with 1% osmium tetroxide and contrast was performed with 0.5% uranyl acetate. A solution of 0.1 M cacodylate buffer was used to wash the tissue between steps. Increasing concentrations of ethanol were utilized to dehydrate the tissue before its inclusion in epoxy resin (Agar Scientific, Essex, UK). The blocks were subjected to semi-thin sectioning (3 μ m), stained with toluidine blue, and analyzed by light microscopy to localize follicles. Thereafter ultrathin sections (70-90 nm) were obtained and analyzed by TEM (Zeiss, Germany). For TEM evaluation, the morphological characteristics of the stroma, granulosa cells and oocytes, as well as their organelles, basal and plasmatic membranes and nuclear envelope, were taken into account. Shape, morphology, distribution and the electron density of chromatin were also investigated.

3. Results

3.1 Histological analysis

Analysis of fresh controls before and after IVC yielded high percentages of morphologically normal follicles: $98.9 \pm 17.7\%$ and $94.5 \pm 5.4\%$ respectively. Some of these normal follicles found in our samples are shown in Figure 2. On the other hand, vitrified-warmed ovarian cortex exhibited signs of degeneration in both follicles and stroma after IVC, as shown in Figure 3. Unfortunately, 100% of follicles were degenerated. They contained oocytes with strong eosinophilic cytoplasmic staining and shrinkage, and either a pyknotic or absent nucleus, confirming their degenerated status. Granulosa cells also showed signs of pyknosis and were arranged in a disorganized way. In tissue, a large proportion of stromal cells showed pyknotic nuclei and fibrotic deposits.

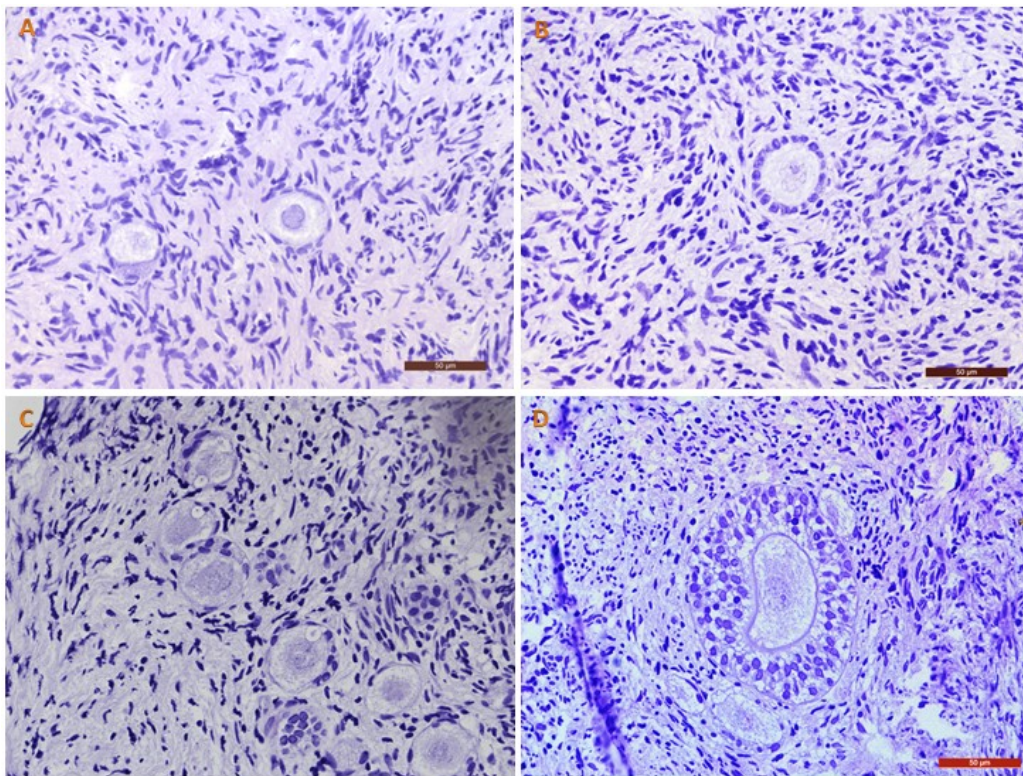


Fig 2 Morphologically normal human ovarian follicles observed in fresh ovarian tissue before (A, B) and after (C, D) 24h of IVC. Bars: 50 μ m

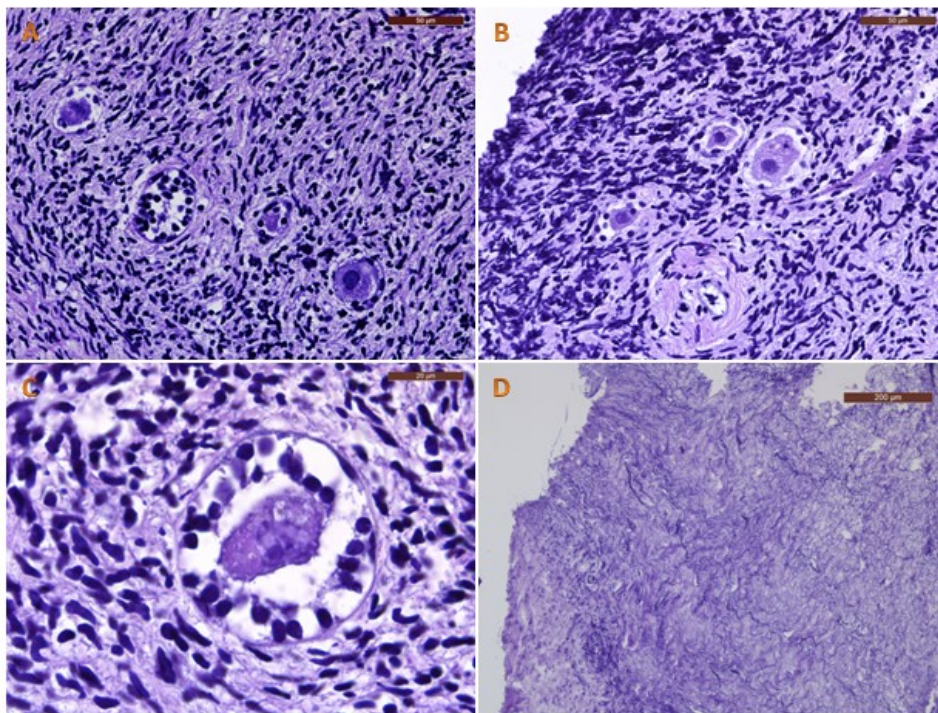


Fig 3 Human ovarian follicles observed after vitrification-warming and 24h of IVC. We can identify degenerated follicles with a shrunken oocyte cytoplasm and pyknotic and disorganized granulosa cells (A-C), as well as fibrotic tissue among stromal cells (D). Bars: 50 μm in A and B; 20 μm in C; 200 μm in D

3.2 X-ray computed tomography

Analysis at -140°C revealed no difference in attenuation between tissue and surrounding medium in two out of three samples evaluated by NanoCT, indicating that the system was well equilibrated (Fig. 4 A-C). In one of the samples, although tissue could barely be distinguished from the solution in the inner part of the vial, the outer region showed higher attenuation close to its border (Fig. 5). This constitutes a normal beam hardening effect, which was corrected with CT calibration. Estimated DMSO concentrations in tissues are shown in Table 1.

Table 1. Average, minimum and maximum DMSO concentrations (% v/v) in tissues analyzed at -140°C and RT, calculated from a VOI of $3 \times 3 \times 1 \text{ mm}$ in the tissue, with calibration curves established for each temperature

Temperature – Patient		Average CT values	Average % v/v DMSO	Min-Max % v/v DMSO
-140°C	P7	2.82 (0.10)	45.2 (± 0.9)	38.0 – 55.0
	P2	2.72 (0.14)	42.0 (± 0.8)	33.2 – 53.2
	P6	2.59 (0.18)	38.1 (± 0.7)	21.4 – 53.1
RT	P7	1.49 (0.05)	0.5 (± 1.0)	-4.8 – 7.3
	P2	1.46 (0.05)	-0.8 (± 1.0)	-6.2 – 4.7
	P6	1.49 (0.07)	0.5 (± 1.0)	-6.4 – 8.4

The average CT error is the standard deviation of the $3 \times 3 \times 1 \text{ mm}^3$ VOI, while average DMSO concentration errors correspond to the standard error of the mean. RT: room temperature; CT: computed tomography; DMSO: dimethyl sulfoxide

After the warming curve was achieved and CPA removal concluded, there was no visible difference between X-ray attenuations of tissue samples and surrounding medium containing 0% DMSO at RT (Fig. 4 D-F). This attenuation similarity led us to conclude that CPA removal was adequate after warming.

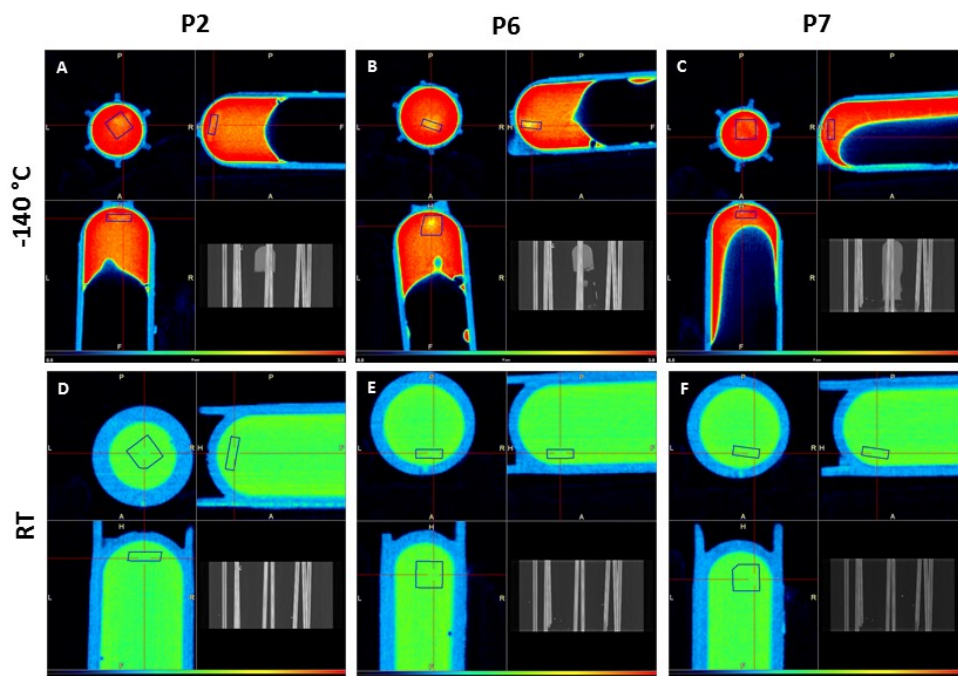


Fig 4 CT images obtained at -140°C and at RT from three different patients (P2, P6 and P7) showing vitrified and warmed ovarian cortex. Spatial resolution is 0.2 mm and the color scale runs from dark blue for low attenuation (1.2 CT, lower concentrations of DMSO) to intense red for high attenuation (3.0 CT, higher concentrations of DMSO). Blue-colored squares shown in all images are volumes of interest (VOIs) located within the tissue to obtain statistical data on attenuation and DMSO concentrations. Tissue can barely be distinguished from surrounding medium in A and C, while in B it can be partly differentiated, showing less attenuated coloration (yellow) than medium containing 50% DMSO (red). After cryoprotectant removal, there is no difference in attenuation levels in the vials at RT. RT: room temperature

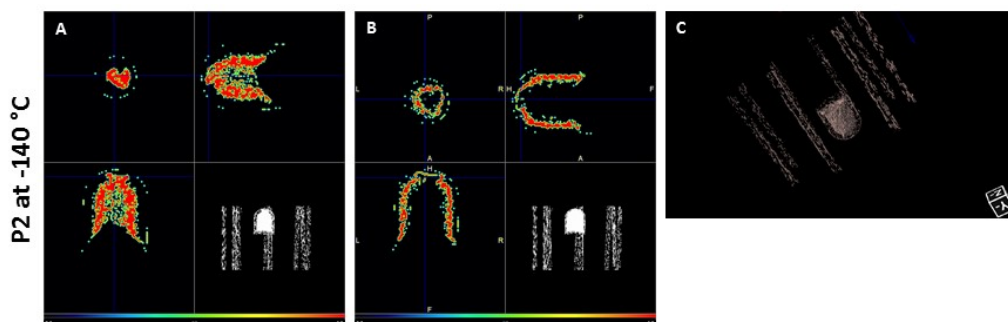


Fig 5 Computed tomography image obtained from one sample at -140°C . A: Segmentation of the CT image showing only part of the picture with a threshold value from 2.6 to 2.8 CT (on a scale of 0.0 to 1.0), which corresponds to a concentration of 38.2% to 44.4% v/v DMSO. The tissue can be distinguished at the bottom of the cryovial with a similar concentration to the medium. B: Image obtained under the same conditions as in A,

but with a threshold value from 2.8 to 3.0 CT, which corresponds to a concentration of 44.4% to 50.6% v/v DMSO. Note the effect at the borders, suggesting higher concentrations than the solution in the middle. C: 3D images obtained with the same threshold value as in A. CT: cycle threshold

3.3 Freeze-substitution

Human ovarian tissue fixed at low temperatures after equilibrium vitrification showed no signs of ice crystal formation, confirmed by normal attachment between cells and no empty spaces in the tissue (Fig. 6 A). In sheep ovarian tissue cryopreserved using an inefficient technique (Fig. 6 B) and serving as a control for comparative purposes, we detected large empty spaces corresponding to ice crystals formed during the freezing procedure.

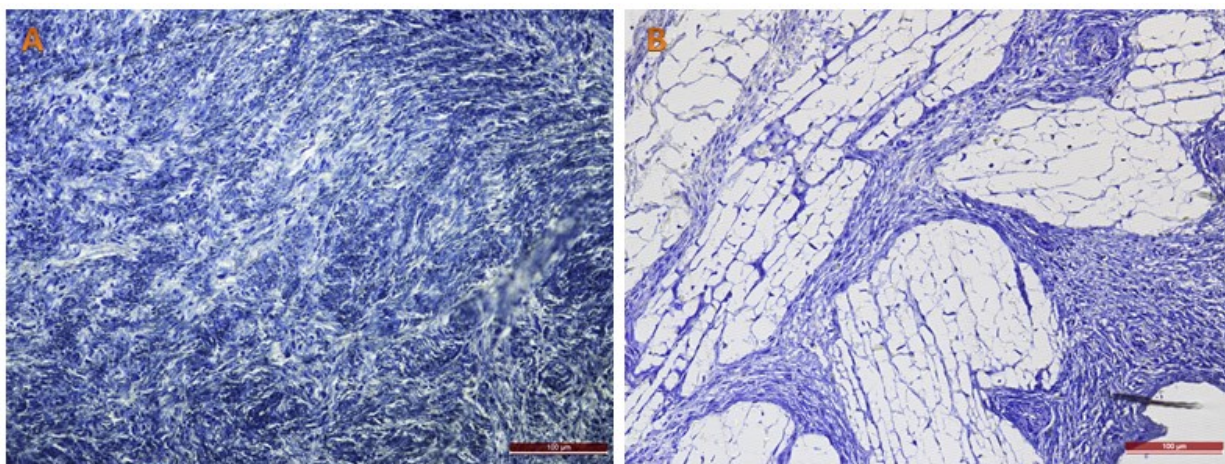


Fig 6 Ovarian tissue fixed by freeze-substitution. Note the absence of spaces between cells and fibers in human ovarian cortex subjected to the stepped vitrification procedure (A), compared to control ovarian tissue subjected to an inefficient cryopreservation procedure (B). Toluidine blue staining; 200x magnification; bars: 100µm

3.4 Transmission electron microscopy

Follicles (n=3) from fresh and vitrified-warmed tissue from three patients were localized and analyzed by TEM. They were all at primordial or primary stages of development.

Fresh tissue contained stromal cells of varying shape, usually with moderately dispersed chromatin. Among these stromal cells, deposits of collagen fiber beams differed from patient to patient, being thicker and more scattered in one subject. Nevertheless, stromal cells from all patients showed signs of morphology maintenance (Fig. 7A). Follicles had a well preserved oocyte (Fig. 7 C) with numerous mitochondria of normal shape (round or cylindrical) and electron density, usually located close to the nuclear envelope (Fig. 7 C and E) and frequently surrounding a small, dense, amorphous granule (Fig. 7 E'). Some lysosomes and small lipid droplets were also present. The cytoplasm exhibited a uniform granular background (Fig. 7E and G), while nuclei showed dispersed heterochromatin and a well preserved nuclear envelope (Fig. 7 E). Adequate attachment was maintained between granulosa cells and oocytes, as well as microvilli (Fig. 7 G). A continuous and intact basal membrane layer between the granulosa and stromal cells evidenced the expected appropriate follicle morphology (Fig. 7 G and H).

Since no preserved follicles were found by light microscopy after IVC, we decided to analyze ovarian tissue ultrastructure immediately after warming in order to identify any alterations typically caused by cryopreservation.

Although cell metabolism and activity were not entirely reestablished, clear signs of degeneration were already visible in cell ultrastructure. It is important to state that during analysis of semi-thin sections by toluidine blue staining under light microscopy, it was difficult to detect signs of degeneration (200x magnification).

Stromal cells showed condensed chromatin, poorly preserved organelles and numerous vacuoles in their cytoplasm (Fig. 7 B). Oocyte organelles were not preserved (Fig. 7 D) and displayed enlarged rough endoplasmic reticulum cisternae, either with flocculent content or in the form of a clear vacuolar structure, sometimes surrounded by detached clustered ribosomes (Fig. 7 F). Mitochondria exhibited clear signs of degeneration, such as disrupted shape, slightly clarified matrices and the remains of cristae (Fig. 7 F). Large vacuoles were also observed throughout the cytoplasm, as was degeneration of cell membranes in terms of detachment and disintegration of the lipid bilayer (Fig. 7 H). The nucleus consistently showed a dense and disorganized chromatin structure, with degeneration of the nuclear envelope and clear signs of pyknosis (Fig. 7 D).

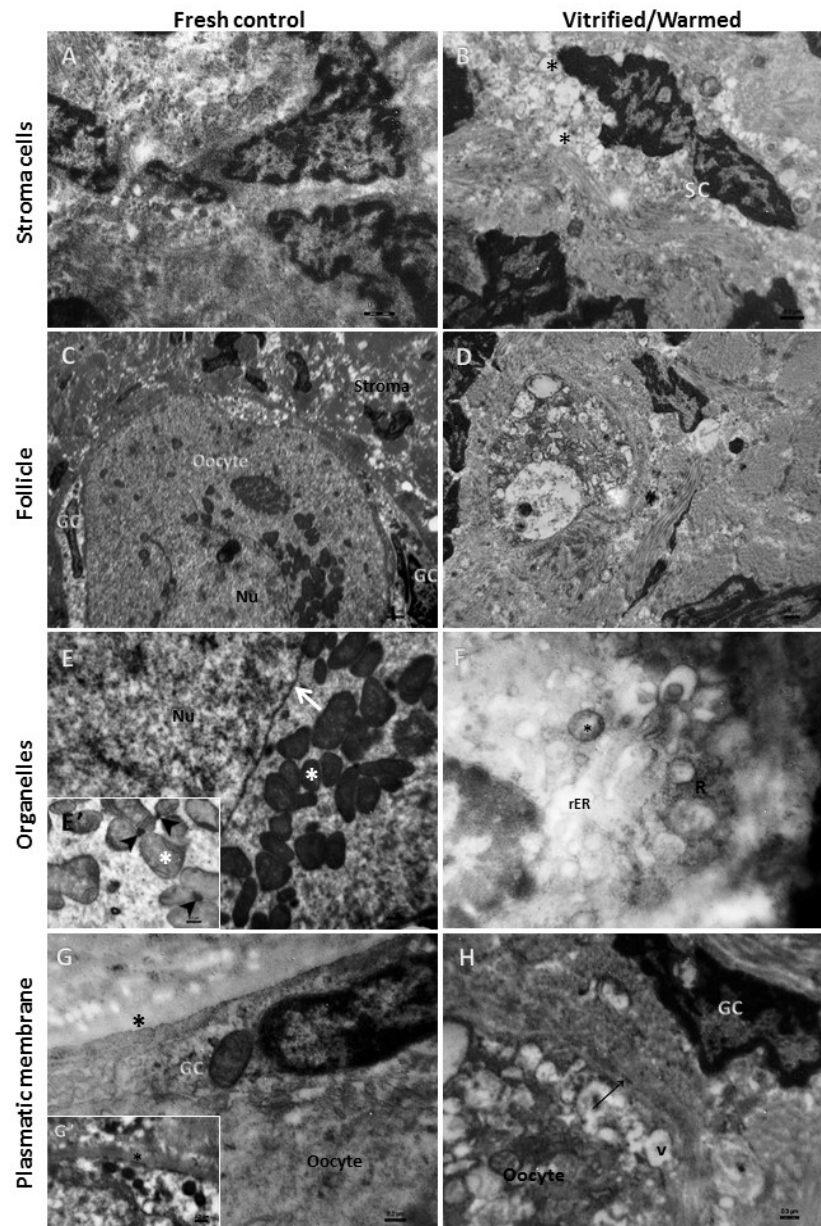


Fig 7 TEM pictures from fresh and vitrified-warmed ovarian tissue. A: Good preservation of stromal cells (3000x); C: Good morphological preservation of cells and attachment between the oocyte and granulosa cells, and the follicle and stroma, in a primordial follicle (700x). E: Well preserved nuclear envelope (arrow) and accumulation of normal mitochondria (white asterisks) around it (7000x). E': Group of mitochondria attached to a dense amorphous granule (arrowheads). G, G': Preservation of oocyte and granulosa cell membranes, with the presence of microvilli and the basal membrane (black asterisks) (4000x). B: Stromal cells showing the presence of several vacuoles in their cytoplasm (asterisks) and a nucleus with highly condensed chromatin (3000x). D: Poorly preserved follicle and an oocyte nucleus with a pyknotic aspect and highly condensed chromatin (400x). F: Presence of degenerated mitochondria (asterisk) and rough endoplasmic reticulum (rER) close to an apparent group of ribosomes (R) (4000x). H: Presence of vacuoles (v) and signs of cell membrane degeneration in the oocyte (arrow) (4000x). SC: stromal cells; GC: granulosa cells; Nu: nucleus

4. Discussion

In this study we evaluated a new vitrification procedure for cryopreservation of human ovarian tissue. However, results from histological analysis were initially discouraging, since no normal follicles were found. We therefore investigated, examining possible reasons why follicle structure was not maintained. Since the main obstacles encountered during cell cryopreservation are ice crystal formation and CPA toxicity, subsequent evaluations were directed at these two possibilities.

CT analysis found DMSO concentrations to be adequate, and the morphological appearance of ovarian tissue after freeze-substitution confirmed no ice crystal formation. A number of alterations to the ultrastructure of follicles were identified by TEM, however revealing that CPA toxicity was responsible for the lack of normal follicles.

Although SF is the most commonly adopted protocol for human ovarian cryostorage today, there is great interest in vitrification systems. Superior results have indeed been obtained with vitrification compared to SF in terms of follicle viability (Desai et al., 2011), DNA fragmentation (Xiao et al., 2013), proportion of intact preantral follicles (Xiao et al., 2013), preservation of stromal cells (Keros et al., 2009), and follicle density and activation rates (Herraiz et al., 2014). A recent review and meta-analysis (Shi et al., 2017) concluded that vitrification may well be more effective than SF, but the authors stressed that variations in protocols could negatively impact the dissemination of the technique and its application in routine clinical practice. It is, therefore reasonable to wonder why a preference to SF still persists. One possible answer is that in addition to the threat of CPA toxicity, direct tissue contact with non-sterile liquid nitrogen could deter practitioners from attempting vitrification (Donnez and Dolmans, 2015). However, this second issue may be avoided by using the Cryotop method (Hasegawa et al., 2004), pooled straws (Vajta et al., 1998) or even cryotubes, as described in the present work, so CPA toxicity remains the main concern.

The effects of this toxicity led us to consider the alternative of SV in humans, which was recently successfully tested for the first time in ovarian tissue (Corral et al., 2018). While results obtained in bovine tissue were promising in terms of follicle morphology preservation, this was not the case in the present study. One of the reasons for the discrepancy may have been that Corral et al. did not proceed to IVC after warming, aware that omission of this step could impair recognition of organelle changes due to toxicity. It is noteworthy that tissue fixed right after warming in the present study did not show obvious signs of degeneration either when analyzed by light microscopy (200x magnification with toluidine blue staining), but it was detected upon ultrastructural analysis. This confirms the need for an incubation step of at least 24 hours of IVC to evidence the impact of the SV method on follicle viability by histology, as previously reported (Mouttham et al., 2015). The opposite findings could also be explained by the heterogeneity in plasmatic membrane composition between different species and types of cells (Depierre and Karnovsky, 1973), since in articular cartilage (Pegg et al., 2006) and encapsulated liver cells (Puschmann et al., 2014), for example, the SV protocol proved extremely efficient at maintaining cell viability and functionality.

During cryopreservation, ice nucleation occurs when the MT is reached, followed by its growth in water molecules, which rearrange themselves in an orderly manner aiming to form a solid crystal (Wowk, 2010). This rearrangement happens wherever water molecules are located, easily destroying tissue components. However, for vitrification to proceed properly in a stepped way, solutes in the systems need to be concentrated/viscous enough to inhibit nucleation (Hunt, 2017). It is known that at -40°C , the viscosity of highly concentrated DMSO solution is increased, while the permeability and fluidity of membranes are decreased (Chapman, 1975), which may impair CPA perfusion. Nevertheless, our CT results showed average equilibrium percentages of DMSO to be between 38% and 45%. According to Wowk (2010), such DMSO concentrations in tissue ensure an absence of ice, preventing crystal

nucleation at -40°C and promoting formation of a vitreous state at -150°C , the temperature of liquid nitrogen vapors. This assumption was corroborated by the freeze-substitution method; since no empty spaces were detected in the ovarian tissue, we had confirmation of adequate CPA equilibrium that promoted appropriate externalization of water and viscosity of the system.

Another important finding was the absence of DMSO in samples after warming. Remains of CPA in tissue have raised concerns because of their potential for damage, such as impacts on epigenetic profiles, as observed in embryoid bodies (Iwatani et al., 2006). After evaluation of residual DMSO in warmed and thawed ovarian tissue, DMSO remnants were only found in vitrified samples (Nakamura et al., 2017), which authors attribute to the fast warming protocol. Since warming was performed slowly in the present study, we achieved total of CPA removal, anticipating no long-term toxic effects on either the potential host or potential embryo.

Once high concentrations of DMSO had been reached and both dehydration and viscosity of compounds deemed adequate, and despite the temperature reduction scheme in the SV method being intended to prevent CPA toxicity, follicle death still ensued. This led us to attribute cell death to chilling injury or high DMSO levels. According to Mazur et al. (1992), chilling injury is thought to increase with exposure time to very low temperatures (Mazur et al., 1992) and its effects are based on membrane phase transition modifications causing membrane disintegration. Reduced CPA toxicity under low temperature condition is related to enzyme activity decreases and lower reaction kinetics in general, be they chemical or osmotic stresses (Puschmann et al., 2014). Since the toxic effects seen in the present study were related to membrane degeneration, they were probably caused by chemical changes in the phospholipid chain conformation that is not attenuated by low temperatures.

Thanks to its capacity to easily cross cell membranes, DMSO is often used in living systems for enhanced penetration of drugs and cryoprotection. It acts by means of three different mechanisms depending on its concentration: (i) by decreasing membrane thickness when used in the range of 2.5-7.5 mol %; (ii) by inducing formation of transient water pores in the range of 10-20 mol %; or (iii) by permanently disorganizing the bilayer structure at concentrations above 25 mol % (Gurtovenko and Anwar, 2007). The cryoprotective properties of DMSO rely on membrane pore formation for externalization of water, but the membrane is unable to recover in concentrations over 25%, even at low temperatures (de Ménorval et al., 2012), as seen in the present study.

Because the toxic effects of DMSO are mostly associated with cell membrane damage (Best, 2015), we assessed toxicity by ultrastructural analysis, which is a widely applied technique (Sheikhi et al., 2013, Fabbri et al., 2016, Talevi et al., 2016) that allows cell membrane evaluation. It revealed extensive damage to organelles and plasmatic membranes. After DMSO penetrates cells, it is distributed throughout the cytoplasm via a microcirculatory tubule system in a directed manner (Kharasch and Thyagarajan, 1983), enabling its spread through cell contents and dissemination to organelles. Mitochondrial alterations were indeed evident. According to Eyden et al. (2004), mitochondria and rough endoplasmic reticulum are the most sensitive organelles in oocytes subjected to cryopreservation. Cell vacuolization was consistently observed during our analysis and is widely reported in the literature; it may be related to swelling and coalescence of smooth endoplasmic reticulum and the Golgi apparatus or plasmatic membrane invaginations due to endocytic vesicles (Sathananthan et al., 1987, Palmerini et al., 2014, Nottola et al., 2016). Irregularly shaped oocyte nuclei and chromatin thickening were also encountered after ovarian tissue cryopreservation (Fabbri et al., 2016). These TEM findings corroborate observations of DMSO toxicity described in the literature: cell membrane disintegration and apoptosis induction (Best, 2015).

In conclusion, our results suggest that rather than preserving human ovarian tissue, 50% DMSO at -40°C causes degeneration of preantral follicles and stromal cells, the concentration being too high. One way of solving this problem could be lowering DMSO levels in the final vitrification solution or even associating CPAs, all at lower concentrations. Indeed, since our objective is to increase viscosity and promote externalization of water from cells, this approach could mitigate membrane disintegration induced by 50% DMSO (de Ménorval et al., 2012).

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