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TRIBUTILESTANHO PREJUDICA CICLO REPRODUTIVO DE RATAS

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RESUMO

Triorganoestânicos, principalmente o tributilestanho (TBT), são contaminantes ambientais organometálicos, comumente usados em tintas anti-incrustantes para embarcações, que sofrem bioacumulação e, portanto, podem ser encontrados em mamíferos e seres humanos, devido à ingestão de alimentos contaminados. A importância do TBT como desregulador endócrino ambiental e sua toxicidade reprodutiva em diferentes modelos animais é bem conhecida. No entanto, os efeitos adversos sobre o ciclo reprodutivo de mamíferos não são bem compreendidos. O potencial tóxico do TBT no ciclo reprodutivo regular de ratas foi examinado. Ratas Wistar com 12 semanas de idade, pesando aproximadamente 230 g foram divididas em dois grupos: Controle (tratadas com veículo, 0.4% solução etanólica) e TBT (tratadas com tributilestanho, 100ng/Kg/dia). O tratamento durou 16 dias. O TBT alterou a regularidade do ciclo estral (%) (de animais com ciclo regular), reduzindo a duração do ciclo, as fases de proestro e diestro, e o número de células epiteliais obtidas no lavado vaginal na fase de proestro. TBT também aumentou a duração do metaestro e o número de células cornificadas nessa fase. O peso dos ovários e os níveis séricos de estradiol diminuíram, e encontramos um significativo aumento nos níveis de progesterona. A análise histológica mostrou células apoptóticas nos corpos lúteos e nas células da camada granulosa dos folículos ovarianos dos animais expostos, bem como a presença de folículos císticos, com elevado número de folículos atrésicos e corpos lúteos. A expressão do receptor de estrogênio alfa mostrou-se reduzida nos tecidos uterinos e ovarianos no grupo TBT. O teste do micronúcleo (MN), usando células de ovário de hamster chinês, demonstrou efeito mutagênico dependente da concentração de TBT. O potencial tóxico do TBT durante o ciclo reprodutivo pode ser atribuído às alterações encontradas no peso ovariano, desequilíbrio hormonal sexual, e prejuízo no desenvolvimento dos folículos ovarianos.

Palavras-chave: Contaminação Ambiental. Tributilestanho. Desregulação Endócrina. Prejuízo Reprodutivo. Ciclo Reprodutivo. Toxicidade.

ABSTRACT

Triorganotins, mainly tributyltin (TBT), are environmental contaminants, commonly used in antifouling paints for boats, which suffer bioaccumulation and thus are found in mammals and humans due to ingestion of contaminated food. The importance of TBT as environmental endocrine disrupter and consequent reproductive toxicity in different animal models is well known. However, the adverse effects upon reproductive cycle are less well understood. The potential reproductive toxicity of TBT on regular reproductive cyclicity of female rats was examined. Wistar female rats at 12 weeks of age, weighing approximately 230 g were divided in two groups: Control (treated with vehicle, 0,4% ethanol solution), and TBT (treated with tributyltin, 100 ng/kg/d). The treatment lasted 16 days. TBT changed cycle regularity (%) (of animals with regular cycle), reduced the cycle duration, the proestrus and diestrus phases, and the number of epithelial cells in the vaginal smears collected during proestrous. TBT also increased the duration of metestrus and the number of cornified cells in this phase. The weight of the ovaries and levels of estradiol in serum were decreased, and we found a significant increase in progesterone levels. Histological analysis showed apoptotic cells in corpus luteum and granulosa cells layer of ovarian follicles treated animals, with cystic follicles, high number of atretic follicles and corpus luteum. The expression of the estrogen receptor alpha was reduced on uterine and ovarian tissues on TBT group. The micronucleus test (MN), using Chinese hamster ovary cells, demonstrated a concentration-dependent mutagenic effect of TBT. The toxic potential of TBT over the reproductive cycle may be attributed to changes found in the ovarian weight, unbalanced levels of sexual female hormones, and impaired ovarian follicles development.

Key words: Environmental Contamination. Tributyltin. Endocrine Disruption. Reprodutive Impairment. Reprodutive Cycle. Toxicity.

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BSA	Albumina soro bovino
CON	Controle
DBT	Dibutilestanho
FSH	Hormônio folículo-estimulante
GnRH	Hormônio liberador de gonadotrofinas
H&E	Hematoxilina e eosina
HPO	Hipotálamo-hipófise-ovário
IMO	International maritime organization
LH	Hormônio luteinizante
MBT	Monobutilestanho
MN	Micronúcleo
PBS	Tampão fosfato-salina
per se	por si só
PVC	Cloreto de polivinila
RE	Receptores de estrogênio
ER-α	Receptor de estrogênio alfa
ER-β	Receptor de estrogênio beta
ROS	Espécies reativas de oxigênio
RP-A	Receptor de progesterona A

RP-B	Receptor de progesterona B
SDS	Dodecil sulfato de sódio
Sn	Estanho inorgânico
TBT	Tributilestanho
TBTA	Acetato de tributilestanho
TBTCl	Cloreto de tributilestanho
TBTOH	Hidróxido de tributilestanho
TBTO	Óxido de tributilestanho
TPT	Trifenilestanho
TPTCl	Cloreto de trifenilestanho

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1 INTRODUÇÃO

nvolvimento de grandes centros urbanos, com intensas e diferentes atividades industriais e portuárias acarreta o despejo indiscriminado de substâncias químicas de naturezas distintas nos corpos d'água, comprometendo a qualidade dos ecossistemas aquáticos (WATANABE et al., 2002) e *per se* nos terrestres. Em baixas concentrações, algumas dessas substâncias são encontradas naturalmente nesse ambiente e quando em excesso tornam-se prejudiciais, como é o caso de hidrocarbonetos aromáticos policíclicos, hidrocarbonetos pesados, metais pesados, agrotóxicos, materiais particulados, e outros (MORON et al., 2006). Não fugindo à regra, os compostos organoestânicos, tal como tributilestanho (TBT), têm sido amplamente utilizados, desde a década de 20, como agente anti-traças, conservantes de madeira, fluido para transformadores e capacitores, agentes de desinfecção de águas de arrefecimento em circulação industrial, biocidas/fungicidas agrícolas, estabilizadores na produção de cloreto de polivinila (PVC) e principalmente em tintas anti-incrustantes ou *antifoulings* para embarcações marítimas em geral (LUDGATE, 1987; FENT, 1996; HOCH, 2001).

Esses compostos químicos sintéticos pertencem a uma classe de poluentes organometálicos, constituídos por um átomo de estanho, que isolado não é propriamente tóxico, mas por estar ligado covalentemente a uma ou mais cadeias orgânicas (hidrocarbonetos), apresentam padrões toxicológicos complexos nos diferentes modelos animais (HOCH, 2001).

Os efeitos tóxicos dependem então da natureza e do número de grupos orgânicos associados ao átomo de estanho. Cabe mencionar que sua ação tóxica máxima, normalmente, ocorre em compostos com três grupos orgânicos, ou trissubstituídos, como o TBT e/ou o trifenilestanho (TPT). Por causa de suas cadeias orgânicas, os compostos organoestânicos são hidrofóbicos, apresentando baixa solubilidade em água, sendo que sua solubilidade depende de fatores como o pH, a força iônica e a temperatura (FENT, 1996; HOCH, 2001). Em solução aquosa, a forma iônica do TBT

encontra-se em equilíbrio com as formas TBTOH (hidróxido de tributilestanho) e TBTCl (cloreto de tributilestanho) e tem valores de pKa que são mais baixos do que as encontradas no ambiente natural (ALZIEU, 1998; RUDEL, 2003). No ambiente marinho, dadas condições normais de pH e salinidade, o TBT encontra-se principalmente na forma TBTOH, sendo que sua biodisponibilidade se torna maior sob tal forma quando o pH é maior que 8 (ALZIEU, 1998; FENT, 1996).

Alarmantemente, A literatura normativa ambiental e científica nos mostra que a partir da década de 60, o cobre deixou de ser o principal componente metálico utilizado em tintas anti-incrustantes, e o controle da bioincrustação foi assumido pelos compostos organoestânicos (HOCH, 2001; MENG et al., 2009). Segundo Bennet (1996), mesmo existindo no mercado marítimo alternativas, como o chumbo, o óxido de cobre e o óxido de zinco (GODOI et al., 2003; YEBRA et al., 2004), a constatada ação biocida dos organoestânicos, em especial a do TBT (DELGADO FILHO et al., 2011), o tornou o principal composto utilizado no mundo em tintas anti-incrustantes. Acreditava-se que o TBT fosse o biocida ideal devido sua fácil degradação, que pode ocorrer pelo uso de ácidos, ação de microalgas e bactérias presentes no ambiente marinho e pela luz ultravioleta, proveniente da radiação solar ao longo da coluna d'água (GADD, 2000; HOCH, 2001; OMAE, 2003). No meio ambiente, normalmente, o TBT se degrada em dibutilestanho (DBT) e monobutilestanho (MBT), sendo que o final da degradação é o estanho inorgânico (Sn), estável. No entanto, conforme já mencionado, a ação do TBT é complexa e depende de diferentes fatores ambientes, pois o nível de toxicidade pode estar relacionado com a sensibilidade da biota, o teor de matéria orgânica no ambiente em questão e tecido animal específico, o tempo de exposição, a concentração liberada, a biodisponibilidade e a persistência destes compostos antes da total degradação (OLIVEIRA; SANTELLI, 2010; PINOCHET et al., 2009) no ambiente natural e/ou artificial.

da euforia da indústria naval, infelizmente, na França, no início da década de 70 surgiu o primeiro relato dos efeitos prejudiciais do uso do TBT em organismos não-alvos (ALZIEU, 1998). A partir de então, vários estudos ecotoxicológicos mundiais

foram e estão sendo conduzidos com a finalidade de comprovar a extrema toxicidade do TBT ao longo dos diferentes níveis tróficos e modelos experimentais. O que caracteriza um verdadeiro desafio, pois ainda em 1991, cerca de 80% dos barcos de grande porte já usavam algum tipo de tinta a base de compostos organoestânicos (IMO, 1999).

À medida que anormalidades ambientais devido a exposição ao TBT foram surgindo, muitos reagiram com certa incredulidade a respeito de seu potencial tóxico. As primeiras regulamentações adotadas no uso do TBT como biocida nas tintas anti-incrustantes surgiram em 1982, pelo Ministério Francês do Ambiente, com a finalidade de reduzir sua utilização (ALZIEU, 1996). No entanto, esta diminuição não foi generalizada, devido à importância econômica dessas tintas para as indústrias que utilizam o transporte marítimo (LIU et al., 1997). A diminuição da biota incrustante, a redução do risco de introdução de espécies exóticas, redução da frequência de docagem, economia de combustível, a redução nos níveis de emissão de dióxido de carbono e de enxofre foram essenciais pra justificar a continuidade da aplicação das tintas à base de TBT nas embarcações (OMAE, 2003; ABEL, 2000).

Ao longo das duas últimas décadas, no entanto,A elevada toxicidade do TBT para uma grande gama de espécies se sobrepôs à sua importância econômica na indústria naval e uma série de restrições foi adotada em vários países. No Brasil, no ano de 1992 em uma conferência realizada no Rio de Janeiro, foi adotado o “Princípio da Precaução” (Agenda 21. Seção 17.22), ficando então acordado a proibição total do uso do TBT a partir de 1º de Janeiro de 2003. Além disso, em 2001, a IMO (International Maritime Organization) adotou um decreto, segundo o qual, tintas com TBT teriam que ser removidas ou seladas dos cascos das embarcações até Janeiro de 2008 (IMO, 1999). Mesmo com a proibição devido a sua alta toxicidade, entre o início da década de 90 até ano de 2003, estima-se que 70% dos navios do mundo ainda utilizavam o TBT sob a forma de macromolécula formada a partir de unidades estruturais diferentes e menores - copolímero (SWENNEN et al., 1997). Os prejuízos relacionados ao uso do TBT ainda são

evidentes devido a sua deposição em sedimentos superficiais do ecossistema aquático e bioacumulação nos organismos.

Baseado em investigações toxicológicas, os compostos organoestânicos possuem ações cito, neuro e genotóxica em diferentes modelos animais de invertebrados (cracas, gastrópodes) e vertebrados (peixes, roedores e homem). Estas ações deletérias são capazes de afetar alguns animais em seu *habitat* natural, ocasionando alterações morfofuncionais macroscópicas, modificando seu comportamento ecológico, afetando sua reprodução e alterando parâmetros relacionados à sua biodiversidade, reduzindo sua adaptação ao meio (KOVALCHUCK et al., 1998). Desta forma são utilizados com bioindicadores ambientais da contaminação/ poluição pelos compostos organoestânicos, como o caso de moluscos gastrópodes (OHHIRA et al., 2003; DELGADO FILHO et al., 2010; GRACELI et al., 2012).

ncipalmente, de sua bioacumulação ao longo das cadeias alimentares, ou alguma outra forma de contaminação da dieta e / ou água, os triorganoestânicos vêm sendo associados a diferentes prejuízos funcionais em mamíferos, como modificação metabólica *in vivo* e *in vitro* (GRÜN; BLUMBERG, 2006; GRACELI et al., 2012), desenvolvimento de fatores de ricos cardiovasculares, principalmente coronarianos (SANTOS et al., 2012), e disfunções reprodutivas (DELGADO FILHO et al., 2011; PODRATZ et al., 2012; GRACELI et al., 2012).

Dentre os efeitos tóxicos causados pelo TBT, um dos mais notáveis foi o desenvolvimento sexual anormal, devido à desregulação endócrina em algumas espécies de gastrópodes (MATTHIESSEN et al., 1995). Este fenômeno é conhecido como imposex, ou seja, uma superposição de órgãos genitais masculinos em fêmeas. Tal masculinização é notória devido o desenvolvimento de pseudo-pênis e canal deferente em fêmeas afetadas (SHI et al., 2005). Além disso, o desenvolvimento do canal deferente pode romper a estrutura e função dos ovidutos (NAKANISHI, 2008). Sendo assim, o imposex é uma síndrome endócrina irreversível até então, que provoca a esterilização das espécies, podendo levar a uma

diminuição considerável nas populações mais sensíveis, tornando-as excelentes bioindicadores de contaminação por organoestânicos em ecossistemas marinhos (COSTA et al., 2008) (Figura 1). Outras investigações demonstraram que o TBT interferiu no metabolismo de hormônios sexuais em caracóis (SCHULTE-OEHLMANN et al., 1995; OEHLMANN et al., 1998).

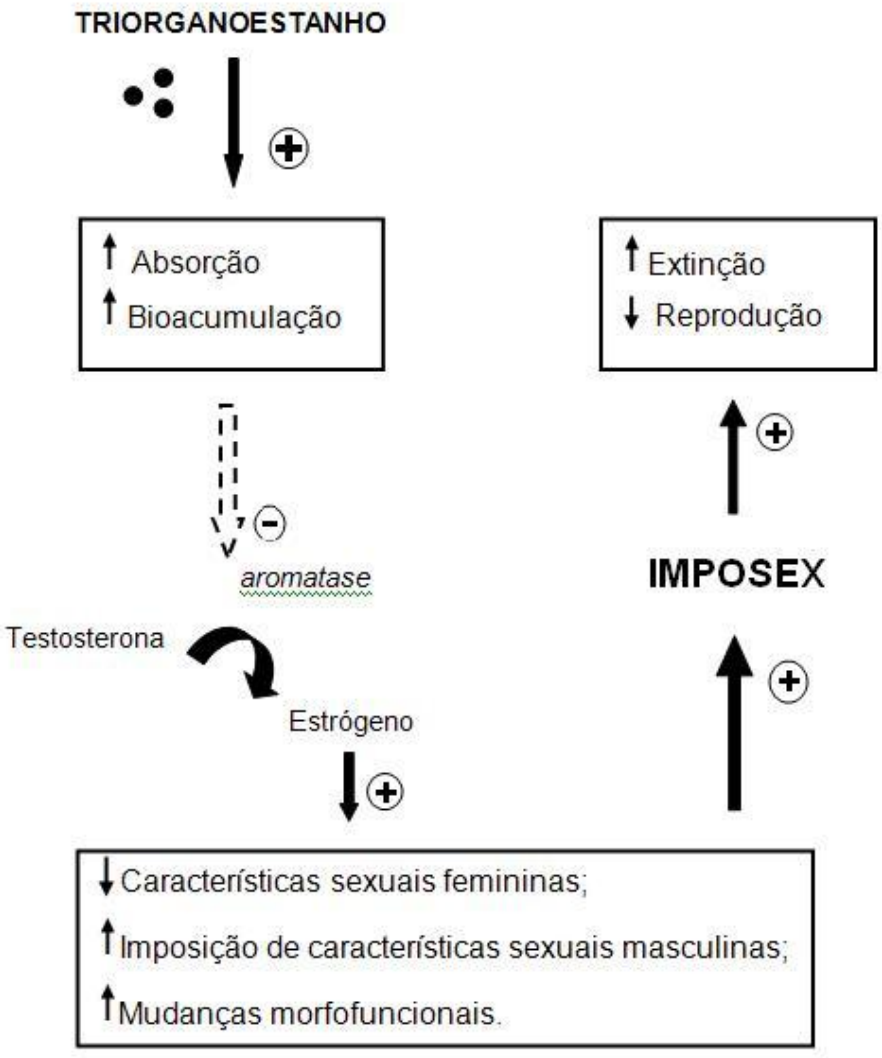

Figura 1 - Diagrama que ilustra o efeito da inibição da enzima aromatase mediada por triorganoestanho, induzindo o imposex. A seta tracejada e as setas contínuas indicam um efeito inibidor (-) e um estimulador (+), respectivamente.

organometálicos, os triorganoestânicos, como o TBT e o TPT, são incluídos ainda nos compostos químicos que possuem ação de desregulação endócrina. pela primeira vez por Theo Colborn e colaboradores, que identificaram uma série de efeitos sobre o desenvolvimento de animais selvagens e seres humanos (COLBORN et al., 1993). desreguladores endócrinos são compostos exógenos com o potencial para alterar a modulação hormonal e do sistema endócrino como um todo, podendo afetar, consequentemente a saúde e a reprodução em animais e seres humanos (USEPA, 2000). Estes compostos podem interferir com a produção, liberação, mecanismos de ação, metabolismo, eliminação, e ainda, podem mimetizar a ação de hormônios naturais (TABB; BLUMBERG, 2006).

Segundo Brown et al. (2001) e Bila e Dezotti (2007), os desreguladores endócrinos podem atuar diretamente sobre uma glândula endócrina, alterando sua função, agir diretamente sobre a expressão de um receptor hormonal e alterar o metabolismo de determinado hormônio em seu órgão-alvo. A exposição aos compostos organoestânicos induz modificações morfológicas, como perda de peso em órgãos reprodutivos (OMURA et al., 2001; OGATA et al., 2001; GROTE et al., 2004), e interferências no metabolismo de hormônios sexuais (OMURA et al., 2001; GROTE et al., 2004; GROTE et al., 2006; KISHTA et al., 2007) em ambos os gêneros de roedores. Sabe-se que hormônios ovarianos, em especial o estradiol, estão envolvidos na regulação das funções reprodutivas e metabólicas (SANTOS et al., 2012). Em mamíferos, o TBT, administrado em diferentes concentrações, é responsável por gerar mudanças na função de uma variedade de tecidos endócrinos, tais como o pâncreas, pituitária, gônadas e a glândula tireoide (WADA et al., 1982; OBERDÖRSTER et al., 1998; VOS et al., 2000).

 podem ser derivados de compostos de origem animal, humana ou vegetal. NGEN) fonteso entanto, a maior preocupação da sociedade ecotoxicológica internacional está focada em produtos químicos sintéticos, tanto os de ação pouco esclarecida, quanto os com capacidade de desregulação endócrina já conhecida (CASALS-CASAS; DESVERGNE, 2011). Preocupação cabível, uma vez que a expansão da indústria química, cuja produção de compostos desreguladores já atingiu 400

milhões de toneladas no mundo, contribui para o aumento da poluição por parte destes produtos químicos, desta forma, provocando um grande impacto sobre a saúde ambiental e humana. Casals-Casas e Desvergne (2011) comprovaram tais impactos, demonstrando que os prejuízos causados pelos desreguladores endócrinos são perceptíveis no desenvolvimento reprodutivo, durante a diferenciação sexual, podem ocorrer em diferentes fases embrionárias e também durante a puberdade, em função de intercorrências na ação dos esteroides sexuais.

A exposição humana aos desreguladores endócrinos, atualmente, pode ocorrer não somente por fontes alimentares, como peixes e mariscos (CHIEN et al., 2002), pela transferência ao longo de cadeias alimentares (KANNAN et al., 1995; IWATA et al., 1995; TAKAHASHI et al., 1999; TANABE, 1999; BILA; DEZOTTI, 2007), mas também por meio da própria água contaminada (LO et al., 2003). Investigações demonstraram a presença de TBT, DBT e MBT em amostras de sangue e fígado humanos (KANNAN; FALANDYSZ, 1997; TAKAHASHI et al., 1999; KANNAN et al., 1999).

o TBT é capaz de alterar a ação das enzimas relacionadas com a esteroidogênese ovariana. Estudos demonstram sua capacidade de inibir competitivamente a enzima aromatase (uma enzima da via do citocromo P450 envolvida na conversão de testosterona em estradiol), o que provoca a diminuição da conversão de andrógenos em estrógenos (OMURA et al., 2001; DELGADO FILHO et al., 2011), aumentando os níveis de testosterona e prejudicando o desenvolvimento sexual de ratos (GROTE et al., 2004). Pode ainda afetar o equilíbrio dinâmico destas reações, acarretando um aumento nos níveis séricos de progesterona (Figura 2). Podendo diminuir o peso uterino, a taxa de gravidez, e número de implantes em ratas pseudográvidas (EMA; MIYAWAKI, 2002). *In utero*, o TBT ocasionou a redução de peso de roedores recém-nascidos, com aumento da frequência de anormalidades esqueléticas (ADEEKO et al., 2003), diminuição do peso materno e do peso dos filhotes, bem como atraso de crescimento e da abertura do olhos na descendência feminina após exposição à dose de 25 µg/Kg de TBTCl (MAKITA et al., 2003). Ainda, em doses mais elevadas, como 10 mg/Kg e 20 mg/Kg, reduziu o número de células

germinativas e gonócitos, afetando o desenvolvimento sexual de ratos (KISHTA et al., 2007). Associado a estas perturbações do sistema endócrino, foi relatada a incidência de tumores induzidos por organoestânicos no testículo e na pituitária de ratos (WESTER et al., 1990; DIAMANTI-KANDARAKIS et al., 2009).

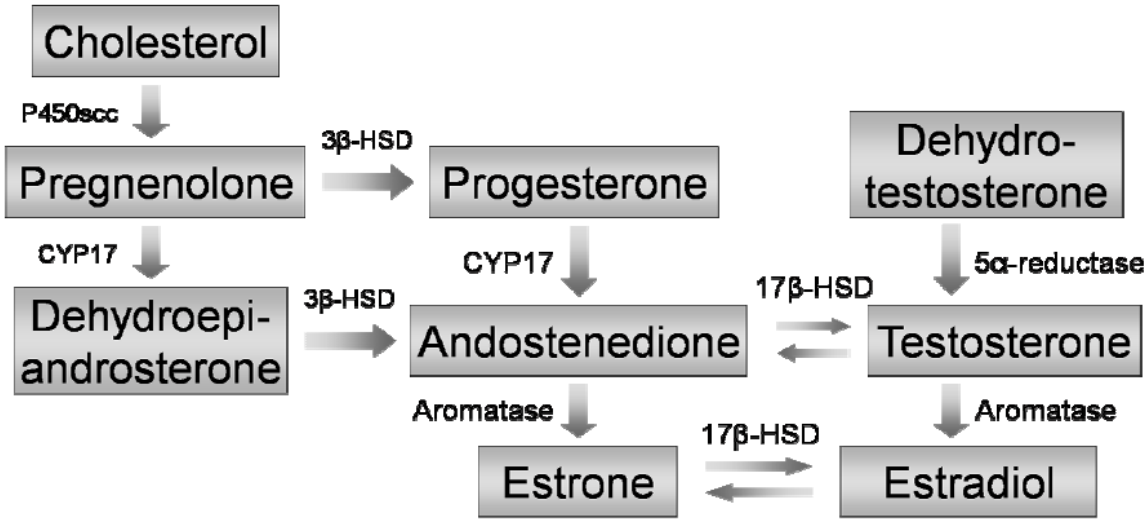

Figura 2 – Esquema da via de biossíntese do hormônio esteróide, onde ações enzimáticas podem ser alteradas por compostos organoestânicos. Figura extraída de Delgado Filho et al., 2010.

A reprodução é um processo fundamental para a manutenção e perpetuação das espécies. Nos mamíferos e outros animais superiores, depende da união dos gametas masculinos e femininos, produzidos pelas gônadas masculina (testículo) e feminina (ovários), respectivamente. Em ambos os sexos, há a necessidade de uma orquestração adequada entre o hipotálamo, hipófise e gônadas. O cérebro age como maestro, em que neurônios hipotalâmicos produzem o hormônio liberador de gonadotrofinas (GnRH), que após alcançar a adeno-hipófise, estimula a produção das gonadotrofinas, hormônio luteinizante (LH) e folículo-estimulante (FSH). Nas mulheres, estimulam o crescimento dos folículos ovarianos, a produção de estrogênio, progesterona, e inibina, que atuam em seus órgãos alvo e na modulação

ao longo do eixo hipotálamo-hipófise. Ademais o LH e FSH induzem a ovulação e iniciam a formação do corpo lúteo (FILICORI et al., 1986; MAGIAKOU et al., 1997).

O eixo hipotálamo-hipófise-ovário (HPO), por meio dos níveis circulantes de gonadotrofinas, estradiol e progesterona, regula as mudanças morfofisiológicas em seus órgãos-alvo, como no sistema reprodutivo, incluindo as tubas, útero, vagina e o ciclo sexual mensal ou menstrual. Tal ciclo é dividido em uma fase folicular, ovulatória e lútea (MAGIAKOU et al., 1997).

A fase folicular se inicia logo após a fase lútea tardia do ciclo reprodutivo anterior, com o início do crescimento folicular e um aumento dos níveis séricos de FSH. Um ou dois dias depois, os níveis de LH tendem a subir, enquanto a secreção ovariana de estradiol se inicia. Entre o sétimo e o oitavo dia anterior ao pico de LH pré-ovulatório ocorre um acentuado aumento na secreção de estradiol ovariano. A elevação do nível sérico de estradiol é acompanhada por uma diminuição de FSH. Ocorre em paralelo um pequeno aumento no nível sérico de progesterona, e em seguida observa-se o rápido aumento no nível de LH, caracterizando o pico de LH ovulatório, momento final da maturação do folículo de Graaf com ruptura entre a 16ª e 24ª hora seguinte. O pico pré-ovulatório de estradiol e a redução da progesterona estão relacionados com a rápida maturação folicular, refletindo no aumento da atividade funcional das células da teca e da granulosa do folículo maduro, destinado a ovular (MAGIAKOU et al., 1997).

Na fase lútea, as células foliculares estão luteinizadas - sofrem hipertrofia, têm sua capacidade de síntese hormonal aumentada, apresentam aumento de grânulos lipídicos, aumento do volume nuclear e do retículo endoplasmático agranular - e o folículo pós-ovulatório se torna cada vez mais vascularizado. O LH é necessário para a sobrevivência e manutenção das funções normais do corpo lúteo durante o ciclo reprodutivo. A característica mais importante desse período é o aumento acentuado da progesterona secretada pelo corpo lúteo. Há um aumento em paralelo no nível de estradiol, mas sabe-se que esse aumento é menor comparado ao da progesterona. O aumento da progesterona ocasiona o declínio de LH e FSH durante a maior parte da fase lútea. Porém, o FSH começa a elevar-se no final da fase lútea

para iniciar o crescimento folicular do ciclo seguinte (YEN, 1978; FILICORI et al., 1986; MAGIAKOU et al., 1997).

Em roedores, como as ratas, o ciclo estral ou ciclo reprodutivo regular tem duração média de quatro ou cinco dias (FREEMAN, 1988; SPORNITZ et al., 1999; BIANCHI et al., 2001) e é caracterizado por quatro fases, proestro, estro, metaestro e diestro (FREEMAN, 1988). Assim como no ciclo menstrual em mulheres, os níveis de LH e FSH começam a aumentar no início da fase folicular, ou pré-ovulatória, o proestro, que é identificado pela presença de aglomerados de células epiteliais nucleadas, e normalmente dura 1 dia. Embora o desenvolvimento folicular ocorra desde o início do proestro (SCHWARTZ, 1964), a fase seguinte, o estro, é que corresponde à fase ovulatória onde a progesterona encontra-se em seu nível máximo. As células da vagina nessa fase apresentam-se rotineiramente cornificadas, com intensa descamação. A predominância de células cornificadas dura 1 dia de um ciclo de 4 dias, ou pode durar 2 dias consecutivos em um ciclo de 5 dias. Na fase seguinte, metaestro, a secreção de estradiol começa a aumentar, atingindo níveis máximos no proestro do ciclo seguinte, retornando à linha de base em estro. A secreção de progesterona também aumenta durante o metaestro, e após o diestro os níveis de progesterona voltam a diminuir. Em seguida, o nível de progesterona aumenta para atingir o seu segundo pico no final do proestro. O termo metaestro é utilizado para descrever um período de transição, ou seja, corresponde à fase entre os ciclos, na qual não há ação máxima de apenas um hormônio, e assim as células vaginais se mostram diversificadas: uma combinação de leucócitos, células cornificadas e células epiteliais. Em seguida, no diestro, as células da vagina não apresentam descamação e há grande número de leucócitos no muco vaginal. Nessa fase o estradiol começa a agir sobre o organismo, mesmo com níveis correspondentes à metade da taxa máxima. Em um segundo dia de diestro, no muco vaginal podem ser observadas células epiteliais nucleadas aglomeradas, que anunciam o proestro do dia seguinte, fase em que a ação máxima do estradiol ocorre (SCHWARTZ, 1964; CARTER, 1993; SPORTNITZ et al, 1999).

Esse controle do desenvolvimento do ciclo reprodutivo via ação esteroidal sexual, desempenhado principalmente pelo estradiol e progesterona, nos diferentes órgãos-alvo é normalmente mediado por receptores hormonais específicos. São eles: os Receptores de Estrogênio (ER) – ER-α (alfa) e ER-β (beta) – geneticamente diferentes (KUIPER et al., 1996; HALL et al., 2001; GUSTAFSSON, 2003), e os Receptores de Progesterona-A (RP-A) e Progesterona-B (RP-B) (LEMALE et al., 2008), que possuem uma vasta distribuição em vários órgãos do indivíduo (KUIPER et al., 1996). Todos esses receptores são membros da superfamília de receptores nucleares, com ações regulatórias variadas na transcrição gênica, no citoplasma e na membrana celular (NADAL et al., 2001). Quando ligados ao hormônio específico, cada receptor sofre rearranjos conformacionais, levando a formação de homo ou heterodímeros de ER-α, ER-β, RP-A e/ou RP-B. Ativados, interagem diretamente com alta afinidade com regiões específicas do material genético da célula, isto é, genes específicos, chamados de elementos responsivos do estrogênio ou da progesterona (LEMALE et al., 2008). Ainda podem atuar indiretamente, como promotores, por meio de interações proteicas com fatores de transcrição (HALL et al., 2001), que ativam e / ou inibem a transcrição gênica.

Embora haja muitas investigações demonstrando os efeitos tóxicos dos organoestânicos em diferentes alvos do sistema reprodutivo, como peso de órgãos sexuais, diferentes fases do desenvolvimento, atividade de enzimas esteroidogênicas, alterações morfopatológicas *in vivo* e *in vitro*, pouco se sabe acerca das possíveis modificações no ciclo reprodutivo de ratas férteis, em função de alterações nos hormônios sexuais induzidas pela exposição ao TBT.

2 OBJETIVOS

2.1 Objetivo geral

O presente trabalho objetiva melhor compreender e avaliarInvestigar as possíveis modificações no ciclo reprodutivo de ratas com ciclo estral regular, em função de alterações nos hormônios sexuais induzidas pela exposição ao tributilestanho.

2.2 Objetivos específicos

- Identificar os efeitos da exposição do tributilestanho no ciclo estral;

- Identificar os efeitos da exposição do tributilestanho na histologia ovariana;

- Identificar os efeitos da exposição do tributilestanho na expressão da proteína do receptor ER-α ovariano e uterino;

Verificar o caráter mutagênico dose dependente do tributilestanho em células ovarianas de Hamster Chinês.

3 METODOLOGIA

3.1 Animais Experimentais e Tratamentos

Foram utilizadas ratas Wistar (*Rattus norvegicus*) pesando entre 200 e 230 g, com 12 semanas de idade, mantidas em gaiolas de polipropileno (43 cm × 30 cm × 15 cm). As condições de temperatura e umidade do ambiente foram controladas, o ciclo de 12 horas de claro / escuro foi mantido, e as ratas tiveram livre acesso à água e comida durante todo o experimento. Todas as ratas apresentaram ciclo estral regular de 4 a 5 dias, sendo que o ciclo reprodutivo foi monitorado por análise do esfregaço de secreção vaginal recolhida diariamente durante pelo menos 2 semanas consecutivas antes de iniciar os experimentos (LISBOA et al., 2001; PODRATZ et al., 2012).

grupo tratado diariamente com tributilestanho, na dose de 100 ng/kg de peso corporal/dia de tratamento. O TBT (Sigma, St. Louis, MO) foi diluído na solução veículo (solução de etanol 0,4%). O tratamento durou 16 dias (para abranger em média 4 ciclos estrais consecutivos), e foi realizado por via oral. (2) Controle (CON, n=10), grupo que recebeu solução veículo seguindo o mesmo protocolo aplicado ao grupo TBT.

Segundo a Agência de Proteção Ambiental dos Estados Unidos (USEPA, 1997), a dose considerada segura ou aceitável para humanos em exposições residenciais ao TBT é de 0.03 mg/Kg por dia. Para se chegar a essa dose é feita a caracterização do risco, avaliando a toxicidade do composto e a relacionando com a exposição à qual o indivíduo é submetido. Para tanto, estudos de dose-resposta são comumente realizados para se conhecer os parâmetros NOAEL e LOAEL de cada composto químico, ou seja, as doses de referência máxima e mínima, respectivamente, que

podem ser administradas sem que haja efeitos adversos no indivíduo exposto ao composto. Usando ainda fatores de incerteza (FI), uma extrapolação de dados animais para humanos é feita durante avaliação do risco, o que fornece uma margem de segurança adicional.

Dessa forma, o cálculo da dose admitida para ingestão diária (IDA) é:

IDA= NOAEL ou LOAEL / FI

Onde FI em estudos animais é 100 (10X para extrapolação inter-específica e 10X para variações intra-específicas) (USEPA, 1997).

A dose selecionada para o tratamento dos animais no presente trabalho é 300X menor do que a considerada segura para ingestão diária de TBT. Essa seleção foi feita para avaliar os possíveis efeitos tóxicos do TBT no grupo tratado, mesmo sob exposição diária aparentemente segura.

Todos os experimentos foram realizados com de acordo com as orientações do *Biomedical Research Guidelines for the Care and Use of Laboratory Animals* (disponível on-line em http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao_879), seguindo as recomendações do *American Veterinary Medical Association Guidelines*, 2007 (disponível on-line em http:\\www.nih.gov). E ainda, Ttodos os procedimentos foram aprovados previamente pelo Comitê de Experimentação Animal da Universidade do Estado do Espírito Santo (CEUA número 047/10).

3.2 Determinação Das Fases Do Ciclo Estral

O ciclo estral foi determinado uma vez por dia por meio de exame citológico do esfregaço vaginal obtido por 2 semanas consecutivas antes e durante os 16 dias de tratamento com o TBT. Tal protocolo adaptado de Marcondes et al. (2002). Toda manhã entre 8:00 e 9:00 horas, cada gaiola contendo os animais foi levada para a sala de experimentos. A secreção vaginal foi recolhida através da inserção (não tão profundamente) de uma ponteira limpa na vagina de cada rata, um volume de 20 microlitros de salina era liberado no interior da vagina e, subsequentemente, o mesmo volume era aspirado. O fluido vaginal misturado à salina foi colocado sobre lâminas de vidro. Uma lâmina de vidro diferente foi utilizada por dia para cada animal dos dois grupos.

O material, que não foi corado, foi observado sob um microscópio de luz (Bel Photonics FLUO-2), sem o uso de lentes do condensador, em aumento de 100X e 400X. As fases do ciclo estral foram determinadas por citologia: quando houve predomínio de células nucleadas epiteliais, a fase foi caracterizada como proestro; se houve predominância de células epiteliais cornificadas, a fase foi caracterizada como estro; quando houve a presença de células epiteliais nucleadas, bem como cornificadas e ainda leucócitos em proporções semelhantes, a fase foi caracterizada como metaestro. E por fim, quando houve o predomínio de leucócitos caracterizamos a fase foi caracterizada como diestro. A frequência total de cada fase observada neste período para cada animal foi utilizado para calcular a duração das fases de proestro, estro, metaestro e diestro (em dias) e a duração do ciclo estral total (AKAMINE et al., 2010; GUERRA et al., 2010; MARCONDES et al., 2002).

A porcentagem de regularidade do ciclo estral foi determinada pela relação: número de ciclos estrais regulares / número de ciclos estrais totais × 100.

O software Image Pro-Plus 4.5.1 (Media Cybernetics) foi utilizado para contar o número de células em cada esfregaço vaginal de cada dia de tratamento a partir de 320 imagens de alta potência captadas (160 imagens do grupo controle e 160 do grupo TBT). Todas as quantificações foram realizadas por um único observador.

3.3 Eutanásia e Dosagem Hormonal

Durante a manhã do primeiro estro apresentado por cada rata, após os 16 dias de tratamento, cada animal foi levemente anestesiado com ketamina (dose de 30 mg / kg de peso corporal, via injeção intramuscular) e xilazina (dose de 3 mg / kg de peso corporal, via intramuscular). Em seguida, cada fêmea foi heparinizada (dose de 100 U / kg de peso corporal, via injeção peritoneal) e eutanaziada 15 minutos após a injeção de heparina.

Após decapitação, as amostras de sangue foram recolhidas e imediatamente centrifugadas a 825 G, a 4 ºC durante 10 minutos para se obter plasma, que foi acondicionado a -20 ºC para medições futuras dos níveis de estradiol, progesterona, testosterona por radioimunoensaio (Diagnostic Products Corporation, Los Angeles, CA) (MOYSÉS et al. 2001).

3.4 Retirada e Pesagem dos Órgãos

Os ovários, útero, glândulas supra-renais e fígado de cada fêmea foram isolados e removidos em fase de estro e em seguida foram pesados (peso úmido) em balança

de alta precisão. As possíveis extensões da hipertrofia dos órgãos foram estimadas para cada animal, calculando a razão do peso de órgãos e o peso corporal (UGGERE et al., 2000).

3.5 Análise Histológica

Os animais, sob anestesia, foram perfundidos com solução salina estéril contendo heparina (10 U / ml) através do ventrículo cardíaco esquerdo. Em sequencia, realizou-se perfusão com formaldeído 10% tamponado com tampão fosfato-salina (PBS-formol). Os ovários foram removidos, fixados em PBS-formol com pH 7,4, por um período entre 24 e 48 horas à temperatura ambiente. Após a fixação, os tecidos foram desidratados em etanol, clarificados em xilol e banhados em parafina a 60 ºC. Posteriormente, os blocos de parafina contendo os tecidos foram cortados em um micrótomo (Leica RM 2125 RTS), gerando cortes de 5 µM de espessura. Os cortes foram corados com hematoxilina e eosina (H&E).

Em cada ovário, os folículos e corpos lúteos foram contados em três cortes por animal e o número foi expresso por unidade de área (mm^2), como descrito por Guerra et al., (2010). Os folículos ovarianos foram posteriormente classificados de acordo com Borgeest et al., (2002) e Talsness et al., (2005). Os folículos ovarianosForam caracterizados como pré-antrais apresentaram de duas a quatro camadas de células da granulosa, sem espaço antral. Os folículos ovarianos foram classificados como antrais quando continham três ou mais camadas de células da granulosa e um espaço antral bem definido. Folículos ovarianos atrésicos são os que apresentaram células da granulosa desorganizadas, com núcleos picnóticos, ovócitos degenerados e desorganizados, e descolamento da membrana basal. Os folículos ovarianos primordiais e primários não foram contados. Em cada corte, 5

regiões diferentes foram analisadas, resultando em um total de 15 contagens por animal.

Para a análise histológica, foi utilizado um sistema de análise de imagem que consistiu de uma câmara digital (Evolution, Media Cybernetics, Inc., Bethesda, MD) ligada a um microscópio de luz (Eclipse 400, Nikon). Imagens de alta qualidade (2048 × 1536 pixels) foram capturadas com o Pro Plus 4.5.1 (Media Cybernetics).

3.6 Extração e Dosagem de Proteínas

Alguns ovários e os úteros retirados durante o experimento foram pesados e armazenados a -80 ºC para a posterior extração de proteínas totais e realização do *western blotting*. O primeiro passo realizado para a extração foi a homogeneização dos tecidos. Para tanto, foi adicionado em cada amostra um volume de 500 µL de tampão de lise contendo 250 mmol/L de sacarose, 1 mmol/L de EDTA, 20 mmol/L de imidazol, pH 7.2, e os seguintes inibidores de protease: 1 mmol/L de fluoreto de 4-(2-aminoetil)-benzenosulfonil, 1 mmol/L de benzamida, 10 mg/L de leupeptina, 1 mg/L de pepstatina A, 1 mg/L de aprotinina, e 1 mg/L de quimostatina. As amostras foram homogeneizadas a 0ºC utilizando um homogeneizador Potter, com 10 incursões por amostra.

As amostras homogeneizadas foram centrifugadas a 10000×*g* por 20 minutos. O pellet foi descartado e o sobrenadante de cada amostra foi armazenado a -80 ºC em tubos eppendorfs para futura quantificação de proteínas totais.

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Para a determinação da concentração de proteínas totais o método de Lowry (LOWRY, et al., 1951), foi utilizado. Um volume de 20 µL do sobrenadante de cada amostra homogeneizada foi utilizado para a dosagem, sendo transferidos para tubos

de ensaio, com adição de tartarato de cobre, reagente de folin e água. Tal método se baseia na reação do cobre com a proteína presente em cada amostra, em meio alcalino, e posterior redução do reagente de folin. Quando ocorre a redução do reagente de folin, o resultado é uma cor mais intensa com absorção máxima em 550 nm, logo, quanto mais proteína, mais intensa é a coloração nos tubos. Para a análise de absorbância foi utilizado o espectrofotômetro.

A concentração de proteínas é determinada por meio de uma curva padrão previamente construída com soluções de diferentes concentrações, e o “branco” utilizado para a construção da curva foi albumina soro bovino (BSA, Sigma-Aldrich).

3.7 Western Blotting (Immunoblotting)

Após as dosagens, retiramos o volume necessário de cada amostra para uma concentração de 100 µg/µL em um volume final de 15 µL. Foi colocado em eppendorfs a quantidade de proteína estabelecida por amostra, adicionou-se água e tampão de amostra contendo Tris–HCl 62.5 mM, pH 6.8, 2% de dodecil sulfato de sódio (SDS), 5% de glicerol, 0.01% de bromofenol azul, e 1.7% de β-mercaptoetanol. As proteínas presentes em cada eppendorf foram solubilizadas, uma vez que os tubos foram colocados em um béquer contendo água a 100 ºC. Os eppendorfs foram mantidos nessa temperatura por 5 minutos.

Conforme Celis (2006), o gel SDS-PAGE (do inglês, *polyacrylamide gel electrophoresis*) foi preparado usando 10% de SDS e poliacrilamida (acrilamida e bisacrilamida). As amostras (15 µL) foram pipetadas cada uma em um poço dos géis e as corridas de eletroforese tiveram duração de 1 hora e 30 minutos cada a 120 Volts. Durante a eletroforese as amostras e os géis se mantiveram imersos em

tampão de corrida. Em seguida, em uma cuba com tampão de transferência, as proteínas foram transferidas do gel para uma membrana de nitrocelulose (Bio-Rad, Hercules, CA).

a transferência, as membranas foram lavadas com tampão tris-salina 0.05% e tween 20 (TBS-T), e bloqueadas com BSA 5% em solução de TBS-T por 1 hora a 4 ºC. Em seguida, as membranas foram novamente lavadas com TBS-T por 10 minutos e incubadas com seus respectivos anticorpos primários, isto é, anticorpo policlonal de coelho para o peptídeo da região C-terminal do receptor de estrogênio alfa de ratos, camundongos e humanos (ER–α, diluição 1:500, em 3% BSA em TBS-T, durante toda a noite a 4 ºC) (Santa Cruz Biotechnology, INC) e com anticorpo policlonal de coelho região C-terminal da β-actina de ratos, camundongos e humanos, sendo a β-actina utilizada como controle interno de cada western blotting realizado (β-actina, diluição 1:1000, em 3 % BSA em TBS-T, durante toda a noite a 4 ºC) (Santa Cruz Biotechnology, INC).

Depois da incubação com os anticorpos primários, as membranas foram lavadas 3 vezes por 10 minutos cada com TBT-T e em seguida, a membrana foi incubada com anticorpo secundário anti IgG de coelho conjugado com fosfatase alcalina (Sigma). Para a incubação do anticorpo secundário, utilizou-se para anticorpo anti ER–α (1:1000, em 3 % BSA em TBS-T, por 1 hora a 4 ºC) (Sigma Immuno-Chemicals) e para o anticorpo anti β-actina (1:4000 em 3 % BSA em TBS-T, por 1 hora a 4 ºC.

O resultado do western blotting de cada amostra foi visualizado pela adição de 200 µL de solução estoque dos reveladores NBT (do inglês, *nitroblue tetrazolium chloride*) e BCIP (do inglês, *5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/mL*) (Life Technologies, Rockville, MD) em 10 mL de solução de tris-HCl e NaCl 0,1 M, pH 9,5, onde as membranas ficaram imersas por 5 minutos, sob agitação em temperatura ambiente. Tais reveladores são utilizados para a detecção colorimétrica da atividade da fosfatase alcalina.

As bandas de ER–α e β-actina foram analisadas por densitometria, por meio de unidades densitométricas arbitrárias, utilizando o Programa Image J. Para a relativa expressão de ER–α, seus valores foram divididos pelos valores correspondentes de β-actina.

3.8 Cultura Celular e Teste do Micronúcleo

células imortalizadas de epitélio ovariano de hamster chinês (CHO-K1; PUCK et al., 1958; KAO; PUCK, 1968) foram cedidas pela Universidade Estadual de Londrina (UEL, Brasil),) e utilizadas para realizar o ensaio antimutagênico do micronúcleo (MN).

Frascos de cultura (25 cm^2) foram utilizados para o cultivo da monocamada de células em meio DMEM / HAM F12, suplementado com 10% de BSA e 0,1% de solução com poder antibiótico e antimitótico.

Os frascos foram mantidos em incubadora BOD a 37 ºC. Sob estas condições, o ciclo das células tem 12 horas de duração. Depois de terem atingido 90% de confluência, as células foram incubadas com DMEM na ausência de BSA durante 12 horas, e posteriormente, foram incubadas com solução de TBT em 3 diferentes concentrações: TBT_1: 2×10^{-3}; TBT_2: 2×10^{-2}; TBT_3: 2×10^{-1} ng/ml, por um período de 3 horas.

Como controle negativo, o tampão fosfato-salina (PBS, pH 7,4) foi utilizado, sem adição de TBT. O agente mutagênico metil metano sulfonato (MMS, 4×10^{-4} M) foi utilizado como controle positivo, sem TBT. A citocalasina (3 ug / ml) foi adicionada

ao meio de cultura com as células CHO-K1 por 18 horas para induzir a formação de células binucleadas.

Os testes do micronúcleo (MN) foram realizados em triplicata. O fixador foi removido antes da diluição das células para a montagem das lâminas com esfregaço celular. Em seguida, as lâminas foram coradas com Giemsa 5% e analisadas de acordo com Malini et al., (2009), onde foram avaliadas mil células por lâmina e contado o número de micronúcleos presentes nas mil células.

Segundo Fenech (2000), para serem considerados micronúcleos, as anormalidades nucleares devem ter morfologia semelhante ao núcleo principal, embora com diâmetro reduzido, não devendo existir conexão física entre eles.

3.9 Análise Estatística

Os dados foram analisados quanto à normalidade e apresentados como média ± erro padrão da média (EPM). As comparações entre os grupos foram analisadas pelo teste t Student (não pareado) e o teste do MN foi analisado por ANOVA (uma via) seguida do teste de Tukey. A significância estatística foi considerada quando $p < 0.05$.

4 RESULTADOS E DISCUSSÃO

4.1 Ciclo Estral, Dosagem Hormonal e Peso dos órgãos

Antes do tratamento com o TBT, os animais dos dois grupos apresentaram ciclo estral regular (Figura 3). O início do tratamento com TBT é representado no gráfico pela seta. Desde o primeiro ciclo estral após o tratamento com TBT foi possível notar o padrão anormal da ciclagem. A exposição ao TBT diminuiu significativamente a regularidade do ciclo estral (%) em quatro ciclos consecutivos.

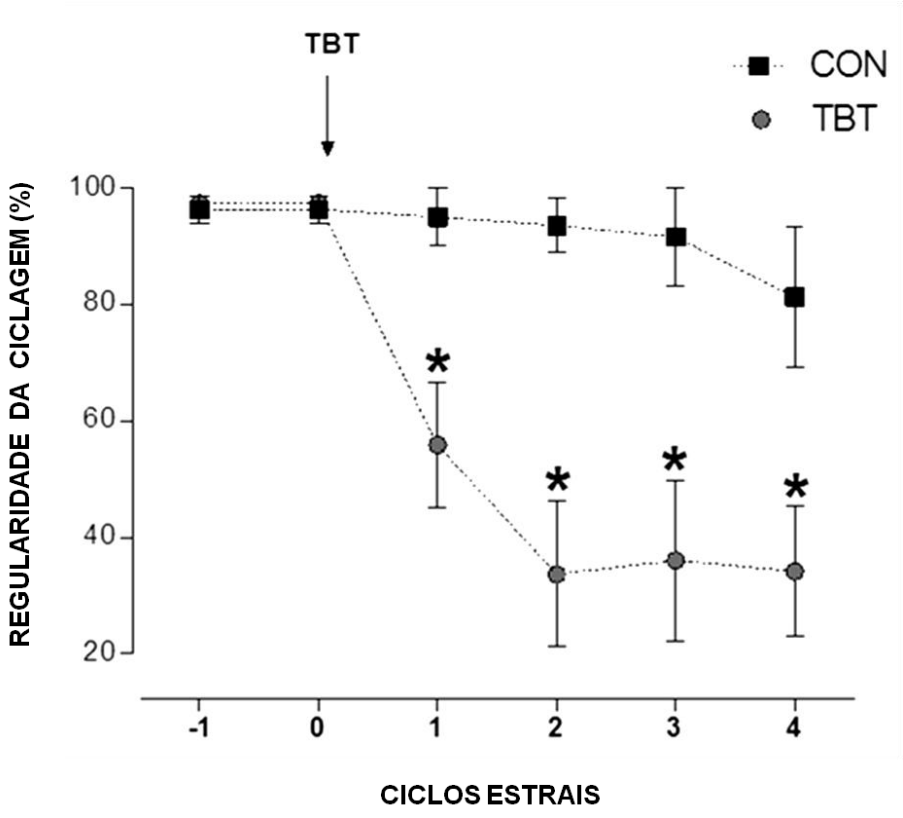

Figura 3 - Regularidade de ciclagem reprodutiva (%) em animais controle (CON) e animais expostos ao TBT. *p<0.05 *vs* grupo controle.

A duração das fases proestro e diestro do ciclo reprodutivo foi significativamente menor, enquanto que a duração da fase metaestro aumentou significativamente (Tabela 1). Apesar deste aumento, houve uma redução na duração total do ciclo estral após administração de TBT.

Tabela 1. Ciclo Reprodutivo: duração das diferentes fases (em dias)

	Controle (n=8)	TBT (n=8)
Proestro	1.1 ± 0.08	0.7 ± 0.12*
Estro	0.8 ± 0.07	0.8 ± 0.09
Metaestro	0.9 ± 0.07	1.3 ± 0.10*
Diestro	1.0 ± 0.08	0.5 ± 0.12**
DuraçãoTotal	3.8 ± 0.11	3.2 ± 0.12*

Valores expressados como média ± E.P.M. * $p < 0.05$; ** $p < 0.01$.

Após análise dos esfregaços vaginais e dosagem hormonal, foi observada uma diminuição significativa no número de células epiteliais durante a fase de proestro (Figura 4) e nos níveis de 17β-estradiol no grupo TBT (Tabela 2). Uma vez que, no ciclo estral regular, o estradiol tem atuação máxima na fase do proestro, pode-se sugerir que a redução hormonal estaria também interferindo na diferenciação celular durante o ciclo reprodutivo. O aumento no nível de progesterona (Tabela 2) e do número de células cornificadas durante a fase de metaestro (Figura 4), sugere que a ação máxima da progesterona em estro se estende ao metaestro, devido à manutenção do seu nível elevado, mantendo assim o número de células corfinicadas elevadas após o tratamento com TBT. Novamente mostrando que a ação hormonal exerce influência na diferenciação celular, justificando as demais alterações encontradas com relação à regularidade (%) e duração das fases do ciclo estral. Diante de tais resultados, tornam-se necessárias novas investigações quanto à histologia da vagina em trabalhos futuros. Visto que o TBT inibe a ação da aromatase, enzima envolvida na conversão de andrógenos em estrógenos, a diminuição de estradiol e o aumento da progesterona eram esperados. Em contrapartida, o fato de não haver aumento no nível de testosterona foi inesperado. Por isso, outras análises também serão necessárias para esclarecer tal resultado. A não alteração no peso do útero, fígado e adrenal após a exposição ao TBT também

não era esperada. A redução do peso ovariano pode sugerir que esse órgão seja mais sensível às alterações macroscópicas do que os demais órgãos, além disso, pode ser mais responsivo à redução de estrogênio que o útero, por exemplo (Tabela 2).

Tabela 2: Concentração de hormônios sexuais femininos e peso dos órgãos retirados

Grupos	OV (mg/g p.c.)	UT (mg/g p.c.)	FIG (mg/g p.c.)	AD (mg/g p.c.)	E_2 (ng/ml)	P_4 (ng/ml)	Testosterona (ng/dL)
CON	11.5 ± 1.0	51.6 ± 0.8	0.94 ± 0.02	0.09 ± 0.02	47.2 ± 7.0	4.0 ± 0.7	4.8 ± 0.8
TBT	8.7 ± 0.9*	51.3 ± 4.2	0.90 ± 0.04	0.10 ± 0.00	32.3 ± 4.3*	7.0 ± 1.2*	4.3 ± 0.2

Valores foram reportados como média ± E.P.M. 17β-estradiol (E_2); Progesterona (P_4); Peso corporal (p.c.); Peso Ovariano (OV); Uterino (UT); do Fígado (FIG); da Adrenal (ADR). *p<0.05 *vs* controle (CON).

Os triorganoestânicos, como TBT e o TPT, mesmo quando administrados em doses menores do que a estipulada como segura pela Agência de Proteção Ambiental dos Estados Unidos (USEPA; 0.03 mg/ kg) (USEPA, 1997), apresentam ação de desregulação endócrina, podendo alterar a regulação hormonal e o sistema endócrino normal, afetando a saúde e a reprodução em modelos experimentais animais e humanos (CASALS-CASAS; DESVERGNE, 2011). É sabido, que os esteróides sexuais influenciam no crescimento, na manutenção, na regulação da função, na regularidade das fases reprodutivas, e na diferenciação dos órgãos reprodutores femininos (GUERRA et al., 2010), justificando a alteração no ciclo reprodutivo, e a diminuição no peso ovariano pela modificação nos níveis de estrogênio e progesterona, após exposição do TBT.

Dentre muitos de seus efeitos nocivos, investigações demonstraram que os organoestânicos prejudicam parâmetros reprodutivos em diferentes modelos experimentais de mamíferos, como demonstrado pelo nosso grupo (DELGADO FILHO et al., 2011), reduzindo também a atividade da aromatase ovariana de ratas

(GROTE et al., 2006), os níveis de estrogênio em células ovarianas da granulosa humanas (SAITOH et al., 2001), aumentando os níveis séricos de progesterona em ratas (EMA; MIYAWAKI, 2002), induzindo a apoptose em células germinativas (KISHTA et al., 2007), e disfunção de órgãos sexuais de ambos os gêneros (OGATA et al., 2001).

4.2 Análise Histológica

Nós encontramos, além das alterações macroscópicas no peso ovariano, modificações morfológicas no tecido ovariano do grupo tratado com TBT em relação ao grupo controle (Figura 5). As ratas do grupo controle apresentaram folículos antrais, corpo lúteo, e estrutura do folículo com aspecto normal. Já as ratas tratadas com TBT demonstraram ovários atrésicos, com presença de folículos císticos e aumento no número de corpo lúteo. Presença de células apoptóticas na área central do corpo lúteo, na camada de células da granulosa e no espaço antral, após exposição do TBT. O tratamento com TBT acarretou um significativo desequilíbrio no desenvolvimento folicular ovariano, como corroborado pelo maior número de folículos atrésicos e corpo lúteo (Tabela 3).

Alterações na regularidade dos picos de estrogênio e progesterona podem provocar efeitos negativos sobre a proliferação e diferenciação celular durante o ciclo estral (BOUTIN; CUNHA, 1997), caracterizando uma disfunção ovariana, causada pelo efeito direto dos organoestânicos no ovário ou por uma modificação dos mecanismos de f*eedback* ao longo eixo hipotalâmico-pituitário-ovariano (GROTE et al., 2006).

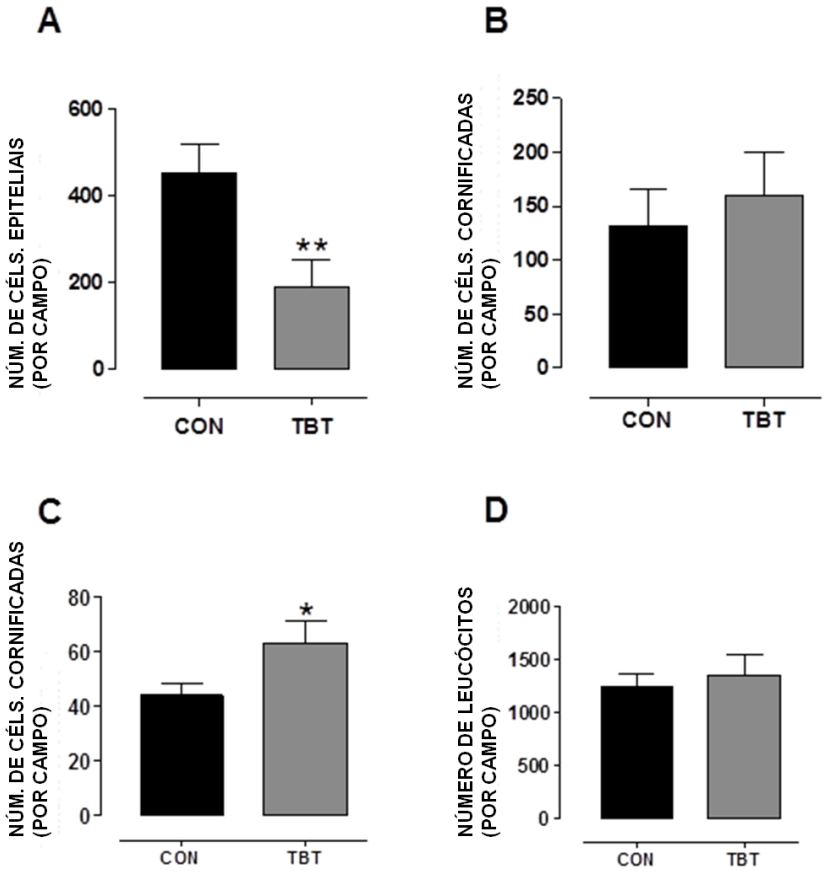

Figura 4 - Contagem de células do lavado vaginal em animais controle (CON) e animais expostos ao TBT. Dados expressos como média ±EPM. (n=8). p<0.05 *vs* grupo controle. A) Fase de proestro: número de células epiteliais na fase de proestro. (B) A fase de estro: número de células cornificadas. (C) Fase metaestro: número de células cornificadas. (D) Fase de diestro: número de leucócitos.

Tabela 3 - Contagem de folículos ovarianos e corpo lúteo por unidade de área (numero/mm^2)

Estruturas	CON	TBT
Folículos Pré-antrais	0.93 ± 0.11	1.10 ± 0.10
Folículos Antrais	0.90 ± 0.10	0.93 ± 0.11
Folículos Atrésicos	0.75 ± 0.10	1.80 ± 0.20*
Corpo Lúteo	0.80 ± 0.09	1.10 ± 0.07*

Valores foram reportados como média ± E.P.M. *p<0.05 *vs* controle (CON).

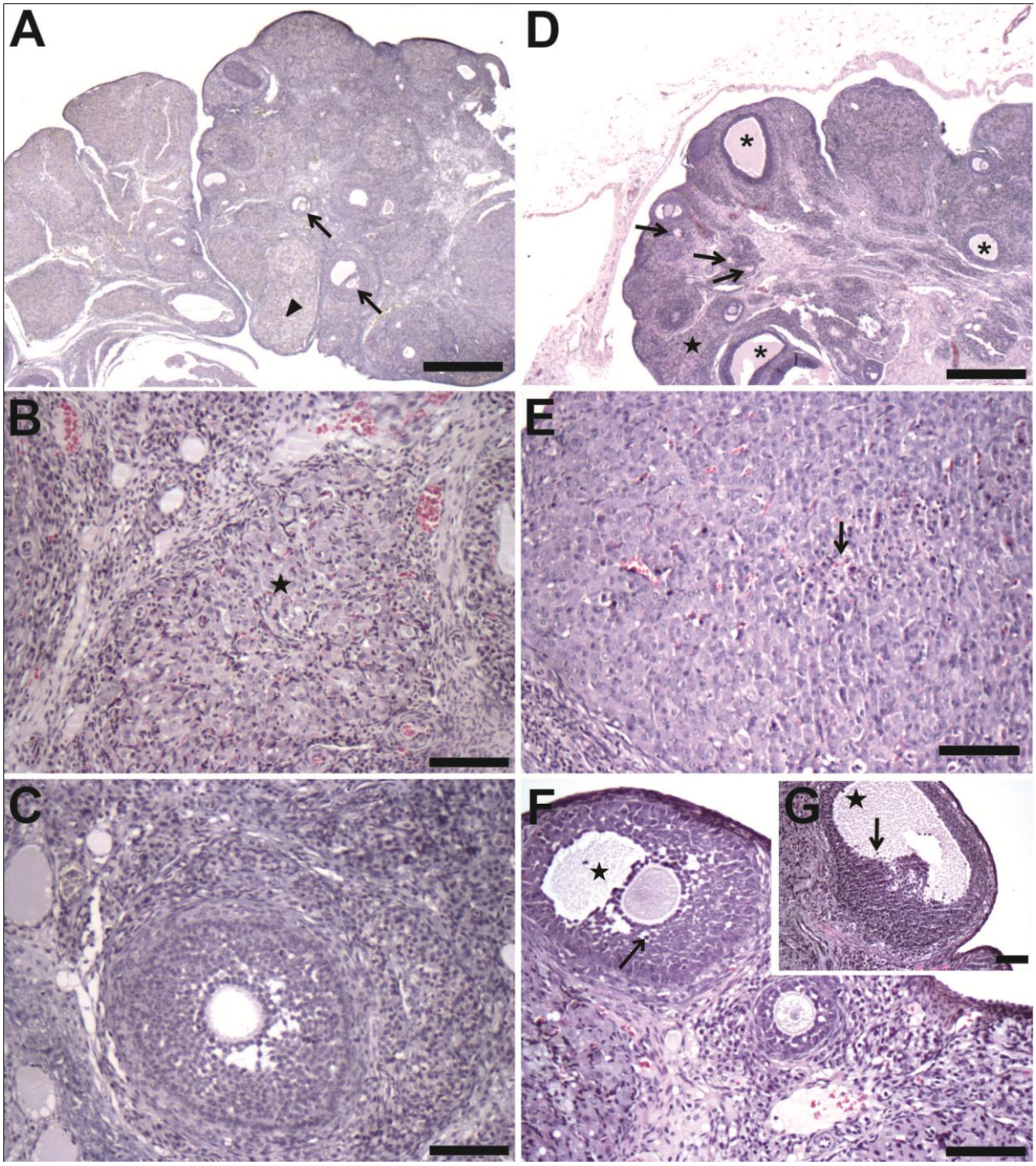

Figura 5 - Análise histológica de tecido ovariano. Grupos CON (A-C) e TBT (D-G)**. (A)** Folículos antrais (seta) e corpo lúteo (cabeça de seta) **(B)** Corpo lúteo (estrela) **(C)** Folículo normal **(D)** Folículos atrésicos (asterisco). Folículos císticos (seta) e corpo lúteo (estrela) **(E)** Corpo lúteo com células apoptóticas **(F)** Folículo antral atrésico (estrela). Células da granulosa mortas (seta) **(G)** Folículo antral atrésico sem oócito (estrela). Células apoptóticas (seta). A, B, D e E (barra: 400 mm). C, F, G (barra: 200 mm).

Esses achados corroboram com o aumento no número de corpos lúteos. Tal aumento é prejudicial, pois é depois da degeneração do corpo lúteo que a concentração de esteróides do sangue diminui e FSH é liberado em quantidades maiores, o que estimula o crescimento de outro grupo de folículos e inicia o ciclo reprodutivo seguinte. Se há um aumento de corpos lúteos, existe o atraso no ciclo seguinte e prejuízo no desenvolvimento dos folículos. Os resultados ainda corroboram com o aumento nos níveis séricos de progesterona, e a presença de células apoptóticas e de folículos ovarianos atrésicos no tecido ovariano das ratas tratadas com TBT. De forma similar, Kistha e demais pesquisadores (2007) demonstraram um aumento no número de células germinativas degenerativas e apoptóticas durante o desenvolvimento ovariano de ratas expostas ao TBT.

De acordo com Sportnitz et al. (1999), o nível de estradiol começa a aumentar em metaestro, sendo que o pico hormonal ocorre durante o proestro e retorna às concentrações basais no estro, enquanto a liberação de progesterona aumenta durante o metaestro e diestro com uma subsequente queda nos níveis séricos. O presente estudo demonstrou redução na duração do ciclo reprodutivo e houve diferenças proporcionais em relação aos tipos celulares que caracterizam as fases do ciclo estral, após exposição ao TBT, sugerindo que a variação proporcional entre as fases é dependente dos níveis de hormônios sexuais. Nossos resultados são corroborados com informações da Agência de Proteção Ambiental Americana (USEPA, 1997), que alega que alterações no peso ovariano podem estar relacionadas à presença de cistos, depleção de folículos ovarianos e ainda a modificações na modulação do hipotálamo e/ou hipófise.

A regulação hormonal do ciclo estral, dos corpos lúteos e do nível de progesterona desempenha um papel importante no controle do ciclo reprodutivo, bem como para a manutenção da gravidez (STOCCO et al., 2007). Peluso (2003) sugeriu que a síntese de progesterona em células da granulosa está intimamente relacionada com a ativação de caspases. Apesar de sua ação mais comum relacionar-se com o processo apoptótico (YUAN et al., 2012), como encontrado em nosso resultados. Ademais a enzima Citocromo P-450scc catalisa a produção de progesterona e gera

um aumento na formação de espécies reativas de oxigênio (ROS) (YACOBI et al., 2007), que exercem um efeito pró-apoptótico, estimulando a capase-3 (NOWICKI et al., 2007), protease de cisteína que, dentre as onze já descritas, desempenha um papel central na condução da via apoptótica efetora (LI et al., 1997). Dados que são corroborados pelo aumento na produção de progesterona associado com sinais de apoptose em células da granulosa de bovinos (HUMMITZSCH et al., 2009).

A administração de pequenas doses de TBT, como no caso do presente estudo (100 ng/kg) apresenta um prejuízo na função reprodutiva. Cooke et al. (2004) registraram diminuição de peso em órgãos como o timo, baço e fígado em filhotes de ratos que sofreram exposição ao TBTCl *in utero* e durante a lactação (doses de 0.025; 0.25 e 2.5 mg/kg). Por outro lado, a administração de doses mais elevadas de TBTO (40 mg/kg, BARONCELLI et al., 1995) (27 mg/kg, FAQI et al., 1997), TBTA (16 mg/Kg, NODA et al., 1991b) e TBTCl (25 e 100 mg/Kg, EMA et al., 1995), observa-se que tais compostos apresentam efeitos tóxicos que promovem alterações viscerais, comprometem a estrutura do esqueleto, e ainda apresentam potencial teratogênico tanto em ratos como em camundongos.

Mais indícios do potencial tóxico do TBT foram Ogata e colaboradores (2001), após exposição de TBTCl na dieta de ratas (5; 25 e 125µg/g de peso corporal), demonstrou um índice de fertilidade foi normal, mas houve redução do número de fetos vivos, e diminuição do peso corporal dos filhotes. Ocorreu também atraso no dia da abertura dos olhos e da vagina nos filhotes. O ganho de peso corporal gestacional também foi comprometido, os animais apresentaram ovários com peso reduzido, houve aumento no peso do útero e ainda apresentaram alterações no ciclo estral.

A toxicidade do TBT é complexa e alguns dados se mostram ainda controversos, em função da modificação dos pesos dos órgãos reprodutivos. Segundo Grote e colaboradores (2006), ratas wistar tratadas nos dias 23 e 33 após o nascimento com o TPT, nas doses de 2 ou 6 mg/ kg de peso corporal/ dia de tratamento, demonstraram pesos ovarianos aumentados. Em contraste, a exposição crônica de

ratos ao óxido de TBT (TBTO) na dose de 50 mg / kg de peso corporal acarretou em uma diminuição no peso ovariano (WESTER et al., 1990). Esses últimos dados são semelhantes aos nossos resultados, uma vez que o peso ovariano do grupo tratado com TBT apresentou redução significativa, associado com o prejuízo na produção estrogênica. Ema e colaboradores (1999) demonstram que não houve diferença significativa no peso do ovário em ratas pseudográvidas previamente tratadas com cloreto de trifenilestanho (TPTCl) nas doses de 3.1; 4.7 ou 6.3 mg / kg de peso corporal.

in utero, puberdade, idade adulta). Estas diferenças toxicológicas nos resultados sugerem a necessidade de mais investigações acerca dos mecanismos pelos quais o TBT está atuando, uma vez que induz o surgimento dos diversos prejuízos reprodutivos apresentados, e pode estar associado a outros que ainda não foram totalmente esclarecidos.

4.3 Western Blotting (Imunobloting)

O TBT, além de produzir danos na regularidade do ciclo estral, provocar disfunções no desenvolvimento folicular ovariano, aumentar o número de folículos atrésicos, corpos lúteos e células apoptóticas e acarretar decréscimo no peso do ovário, causou desequilíbrio na concentração de hormônios sexuais ovarianos.

Além disso, a análise do western blotting demonstrou uma significativa redução da expressão da proteína do ER–α nos ovários e no útero das ratas tratadas com TBT, em relação ao controle.

Tabela 4 - Valores densitométricos arbitrários da expressão da proteína do receptor de estrogênio-α

Expressão de ER-α	CON	TBT
Ovário	1.00±0.03	0.72±0.09
Útero	1.0±0.02	0.90 ± 0.02

Dados expressos como média ±EPM. (n=5). *p<0.05 *vs* grupo controle.

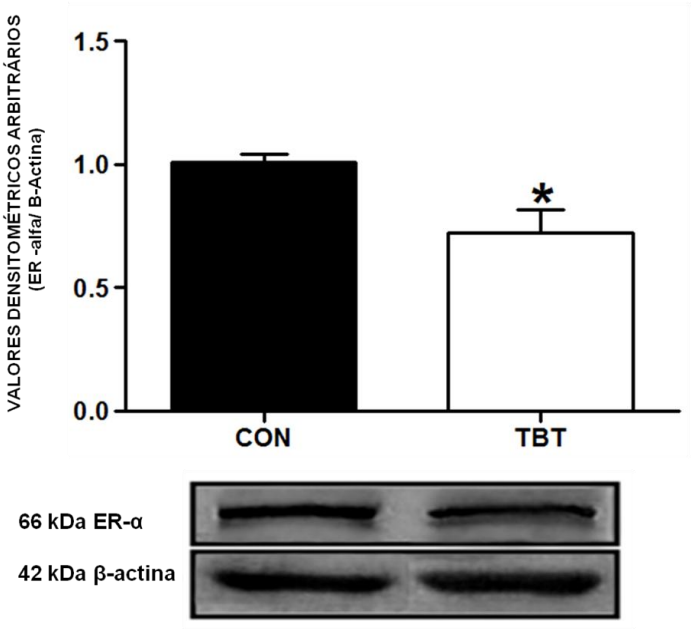

Figura 6 - Expressão do receptor de estrogênio-α no ovário em animais controle (CON) e animais expostos ao TBT. Dados expressos como média ±EPM. (n=5). p<0.05 *vs* grupo controle.

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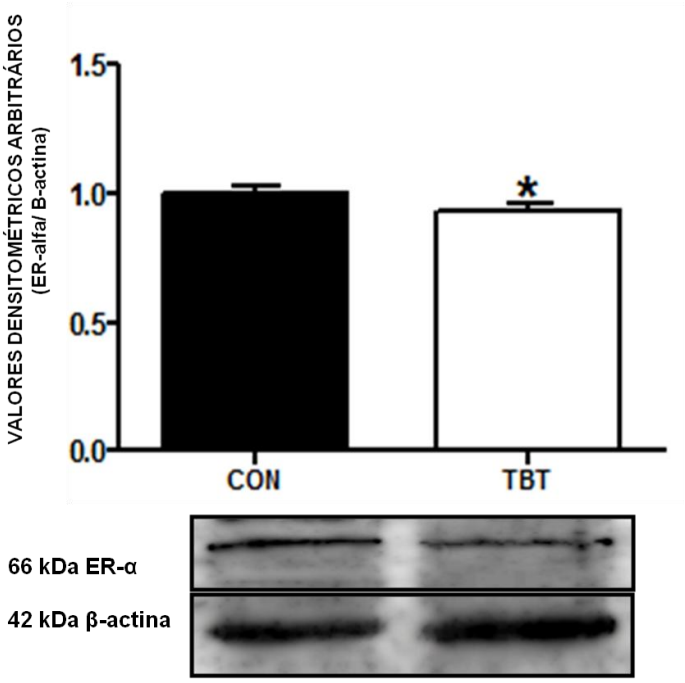

Figura 7 - Expressão do receptor de estrogênio-α no útero em animais controle (CON) e animais expostos ao TBT. Dados expressos como média ±EPM. (n=5). $p<0.05$ *vs* grupo controle.

Como já se A conversão de androgênios em estrógenos é catalisada pela atividade da enzima aromatase em áreas específicas do cérebro, das gônadas, do tecido adiposo, e em vários outros tecidos (GROTE et al., 2006). Há um aumento nos níveis de andrógenos em modelos experimentais de animais invertebrados, como os moluscos (MATTHIESSEN; GIBBS, 1998; OEHLMANN et al., 1996; RONIS; MASON, 1996), em mamíferos, como os ratos (GROTE et al., 2006), e modelos de estudos *in vitro* que constataram a inibição da atividade da aromatase humana (COOKE et al., 2002; HEIDRICH et al., 2001) após a exposição aos organoestânicos.

Baseado em estudos bioquímicos, os compostos organoestânicos podem ser agonistas para receptores nucleares, como o receptor retinóide X (RXR) e receptores PPARγ (do inglês, peroxissoma proliferator activated receptor) (GRÜN; BLUMBERG, 2006). Tanto RXR, quanto PPARγ, quando ativos, são capazes de suprimir a expressão do gene da aromatase no ovário (FAN et al., 2005; MU et al., 2001), através fator nuclear *kB*. (FAN et al., 2005). Em contraste, Nakanishi e seus

colaboradores (2002) observaram um aumento significativo na atividade da enzima aromatase em células de coriocarcinoma da placenta após o tratamento com TBT e TPT.

Nossos resultados indicam que as ratas expostas a baixas doses de TBT também podem ser mais suscetíveis aos distúrbios hormonais, apresentando um prejuízo na da expressão da proteína de receptores de hormônios sexuais, como o estrogênio alfa (Figuras 6 e 7). A redução da expressão de ER–α pode estar relacionada com a falha na produção de estrogênio, devido à exposição ao TBT.

Nos sistema reprodutor femininos há a expressão dos receptores de estrogênios em duas isoformas ER-α e ER-β (KUIPER et al, 1996;. CHU e FULLER, 1997;. PETERSEN et al, 1998). O ER-β é a forma predominante no ovário (BYERS et ai, 1997,. DRUMMOND et al, 1999a) e durante o desenvolvimento pós-natal, há um aumento da expressão de seu RNAm em sinergia com a proliferação das células da granulosa de ratas. A expressão do RNAm do ER-α permanece estável após a sua indução inicial (DRUMMOND et ai., 1999b), indicando de um perfil de expressão proteica mais generalizada no ovário (SAR e WELSCH, 1999; SAUNDERS et al, 2000;. PELLETIER et al., 2000).

As fêmeas que são ERKOs (não expressam o ER-α) apresentam ciclos reprodutivos acíclicos, são inférteis, apresentam ovários hiperemiados e ainda são desprovidos de corpo lúteo. A sua foliculogênese é prejudicada, apresentando um desenvolvimento folicular que fica estagnado com folículos ovarianos antrais bem desenvolvida, tornando se folículos císticos e hemorrágicos (LUBAHN et al, 1993;. COUSE et al, 1997).

4.4 Teste do Micronúcleo (MN)

A análise i*n vitro* com o teste do MN em células CHO-K1 demonstrou o efeito mutagênico dose dependente do TBT, onde a maioria das células expostas ao TBT_3 (2.0×10^{-1} ng/ml), ao final das 3 horas de exposição, estava em suspensão, apresentando-se inviáveis para a contagem de micronúcleos (Figura 8).

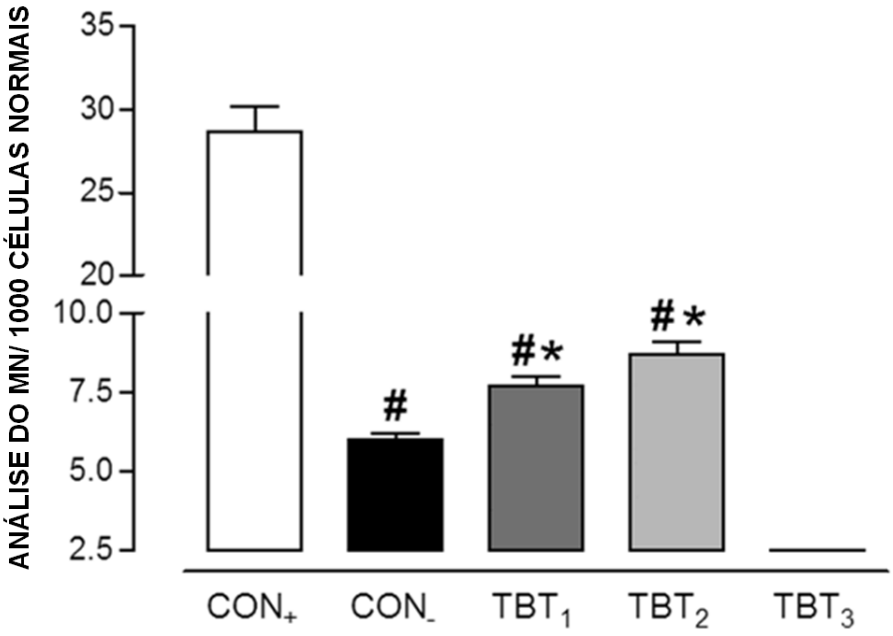

Figura 8. Valores basais de parâmetros mutagênicos (micronúcleo teste) em células CHO-K1 após exposição com diferentes doses de TBT (TBT_1: $2.0. 10^{-3}$; TBT_2: $2.0. 10^{-2}$; TBT_3: $2.0. 10^{-1}$ng/ml). CON-: tratamento com PBS sem presença de TBT. CON+: tratamento com MMS (agente mutagênico) sem a presença de TBT. * $p < 0.05$ comparado com CON- e # $p< 0.05$ comparado com CON_+ (Anova uma via, seguida do teste de Tukey, n = 5). Valores expressos como média ± EPM.

in vitro comprova, o potencial tóxico de compostos organoestânicos (NAKANISHI et al., 2002; NAKANISHI et al., 2006; SAITOH et al., 2001). Triorganoestânicos, como o TBT e o TPT induziram a apoptose e/ou necrose de células de mamíferos, linhagem

celular humana semelhante a células tumorais da granulosa, KGN (NAKANISHI et al., 2002; NAKANISHI et al., 2006; SAITOH et al., 2001).

A proliferação celular, por exemplo, foi suprimida na linhagem celular humana semelhante a células tumorais da granulosa após uma exposição a 200 ng / ml de TBT durante 48 horas (SAITOH et ai., 2001). A exposição de células de embriões de peixes, no período de gástrula ao TBT (10 ng / L) por 144 horas, estimulou a apoptose celular (ZHANG et al., 2011). Ademais, o TBT pode ser responsável pela ativação de caspases e pela consequente apoptose em hemócitos de invertebrados (CIMA; BALLARIN, 1999). Em mamíferos, o TBT induz apoptose na linhagem de hepatócitos humanos Hut-78 (READER et al., 1999), bem como na linhagem celular de linfócitos T-Jurkat (STRIDH et al., 1999), demonstrando seu efeito tóxico para o sistema imunológico.

toxicológicos corroboram com nossos dados de análise do micronúcleo dose dependentes em células CHO-K1, sugerindo um efeito mutagênico após a exposição ao TBT nestas células ovarianas.

5 CONCLUSÕES

As evidências encontradas neste trabalho indicam que, sob determinadas condições experimentais, a exposição a baixas doses de TBT compromete a regularidade do ciclo reprodutivo de ratas, em função de um desequilíbrio nos níveis de hormônios sexuais ovarianos, em parte por prejuízos histomorfológicos e de expressão gênica, dos receptores de estrogênio alfa nos tecidos ovarianos e uterinos. *In vitro*, o MN demonstra um potencial efeito tóxico do TBT em células CHO-K1, sugerindo como um futuro fator de risco para o ciclo reprodutivo. Portanto, a caracterização dos mecanismos envolvidos no surgimento de tais prejuízos pode levar a uma melhor compreensão dos possíveis efeitos do TBT em mulheres e, talvez, proporcionar a base para o desenvolvimento das melhores formas de terapia.

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7. ANEXOS

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Tributyltin Impairs the Reproductive Cycle in Female Rats

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TRIBUTYLTIN IMPAIRS THE REPRODUCTIVE CYCLE IN FEMALE RATS

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Triorganotins are environmental contaminants, commonly used in antifouling agents for boats, that bioaccumulate and thus are found in mammals and humans due to ingestion of contaminated seafood diets. The importance of triorganotins as environmental endocrine disruptors and consequent reproductive toxicity in different animal models is well known; however, the adverse effects on reproductive cycle are less well understood. The potential reproductive toxicity of tributyltin (TBT) on regular reproductive cycling of female rats was examined. Wistar female rats (12 wk old, weighing approximately 230 g) were divided into two groups: control (vehicle, ethanol 0.4%) and tributyltin (100 ng/kg/d, 7 d/wk, for 16 d by gavage). Tributyltin significantly decreased the cycle regularity (%), duration of the reproductive cycle, the proestrus and diestrus phases, and number of epithelial cell in proestrus phase. TBT also increased the duration of metestrus and the number of cornified cells in this phase. Ovary weight and serum 17β-estradiol levels decreased markedly, accompanied by a significant increase in progesterone levels. Histological analysis showed apoptotic cells in corpus luteum and granulosa cells layer, with cystic follicles after TBT exposure. Tributyltin also elevated number of atretic follicles and corpoa lutea. The micronucleus (MN) test, using Chinese hamster ovary cells, demonstrated a concentration-dependent mutagenic effect of TBT, and at 2.0×10^{-2}ng/ml most of the cells were nonviable. The toxic potential of TBT over the reproductive cycle may be attributed to changes found in the ovarian weight, unbalanced levels of sexual female hormones, and number of ovarian follicles and corpora lutea.

Organotin compounds (OT), such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as biocides, agriculture fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters, as well as antifouling paints for marine vessels (Fent 1996). There are many reports of the adverse effects of OT on eukaryotes. One of the most notable toxicities reported was abnormal sexual development and reproduction mediated by TBT and TPT due to endocrine disruption in some species of gastropods (Matthiessen et al. 1995). This phenomenon is known as ''imposex''—the superimposition of male genitalia on female animals. Gastropods are therefore excellent bioindicators of OT pollution in marine ecosystems (Costa et al. 2008). Ecotoxicological investigations also demonstrated that TBT interfered with sex hormone metabolism of snails (Schulte-Oehlmann et al. 1995; Oehlmann et al. 1998).

Organotins (OT) are also suspected to produce endocrine-disrupting effects in mammals, including humans (Golub and Doherty 2004; Kannan et al. 1995), due in part to the possibility of transfer through marine food chains

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(Tanabe 1999). Regarding the effects of TBT toxicological investigations demonstrated that this compound interferes with the sexual hormone metabolism of both gender of rodents (Grote et al. 2004; 2006; Kishta et al. 2007; Omura et al. 2001). TBT inhibited aromatase (Omura et al. 2001), increased testosterone levels, which subsequently disrupted sexual development of male rats (Grote et al. 2004), which led to reduced birth weight, increased frequency of skeletal abnormalities in female gravid rats in utero (Adeeko et al. 2003), delayed growth and eye opening in female offspring (Makita et al. 2003), and at the highest doses reduced the number of gonocytes and germ cells, affecting rats' sexual development (Kishta et al. 2007). Other data showed that OT produced changes in the weights of testes (Omura et al. 2001) and ovaries (Ogata et al. 2001), and decreases in uterine weight and serum progesterone levels, pregnancy rate, and number of implantations in pseudopregnant rats (Ema and Miyawaki 2002). TPT decreased ovarian aromatase activity in female pup rats at postnatal day 33 and 53 (Golub and Doherty 2004; Grote et al. 2006) and suppressed aromatase activity in the human ovarian granulosa-like tumor cell line (Saitoh et al. 2001). In addition, OT also induced tumors in testis and pituitary, and investigators postulated that the tumors resulted from endocrine disruption (Diamanti-Kandarakis et al. 2009; Wester et al. 1990).

Despite all these changes produced by OT, there are few studies that examined the adverse effects of OT on the reproductive cycle of female rats. The estrous cycle is characterized as proestrus, estrus, metestrus, and diestrus (Freeman 1988). Ovulation occurs from the beginning of proestrus to the end of estrus (Schwartz 1964) and the mean cycle length in the female rat is 4 d (Freeman 1988). This short cycle length makes the rat an ideal animal for investigation of changes occurring during the reproductive cycle (Bianchi et al. 2001; Spornitz et al. 1999). Nevertheless, little information is available concerning the impact of hormonal changes during estrous cycle in female rats exposed to OT. There is no apparent study on the effects of TBT on the cycle of the female rats. This study was done to better understand the interactions of OT with the female reproductive system and metallic influences during the reproductive cycle.

MATERIAL AND METHODS

Experimental Animals and Treatments

Adult female Wistar rats weighing 200–230 g (12 wk old) were kept in polypropylene cages (43 cm × 30 cm × 15 cm) with controlled temperature and humidity, with 12-h light/dark cycle with free access to water and food. All female rats showed a regular 4- to 5-d estrous cycle monitored by vaginal smears collected each morning for at least 2 consecutive weeks before starting the experiments (Lisbôa et al. 2001). Rats were divided into two groups: (1) TBT ($n = 10$), treated daily with tributyltin (100 ng/kg/d TBT diluted in ethanol solution 0.4% as vehicle; Sigma, St. Louis, MO, Pcode: 101057974, product reference: T50202-100g, lot: STBB5068V) for 16 d by oral administration; and (2) Control (CON, $n = 10$) received vehicle following the same protocol as applied to TBT group. All experiments were carried out with female Wistar rats in accordance with the Biomedical Research Guidelines for the Care and Use of Laboratory Animals (available on line at (http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao_879), and followed the recommendations of the American Veterinary Medical Association Guidelines, 2007 (available online at http:\\www.nih.gov). All procedures were approved by the Committee for Animal Experiments of the University of Espirito Santo (CEUA number 047/10).

Estrous Cycle Phase Determination

Estrous cycle was determined once a day by cytological examination of vaginal smears obtained for 2 consecutive weeks before starting the experiments and during 16 d of

treatment with TBT as described by Marcondes et al. (2002). Every morning between 8:00 and 9:00 a.m. each animal cage was carried to the experimental room. Vaginal secretion was collected with by inserting the tip into the rat vagina (not deeply), releasing saline, and subsequently aspirating the same volume. Vaginal fluid was placed on glass slides. A different glass slide was used for each cage of animals. From each rat one drop was collected with a clean tip. The unstained material was observed under a light microscope (Bel Photonics FLUO-2), without the use of the condenser lens, with 10× and 40× objective lenses. The estrous cycle phase was determined by cytology: predominance of nucleated epithelial cells (proestrus); predominance of cornified epithelial cells (estrus); the presence of cornified and nucleated epithelial cells and leukocytes (metestrus); and predominance of leukocytes (diestrus). The total frequency of each phase for every rat observed in this period was used to calculate the total length of the proestrus, estrus, metestrus, and diestrus (in days) and the estrous cycle length (Akamine et al. 2010; Guerra et al. 2010; Marcondes et al. 2002). The percent estrous cyclicity was determined: number of regular estrous cycle/number of estrous cycle × 100. The Image pro-plus 4.5.1 software (Media Cybernetics) was used to measure the number of cells from vaginal smears of 320 captured high-powered images (160 from control group and 160 from TBT group) from each daily vaginal smear in the different experimental groups. All quantifications were performed by a single observer.

Collection and Weighing of Organs

The animals were lightly anesthetized with ketamine (30 mg/kg, im) and xylazine (3 mg/kg, im) to obtain target organs at the end of 16 d of treatment. The ovaries, uterus, adrenal glands, and liver were removed in estrus phase and weighed. The extents of organs' hypertrophy was estimated for each animal by calculating the ratio of organs weight and body weight (Uggere et al. 2000).

Hormonal Measurement

Immediately prior to removing the target organs, female rats were injected with heparin (100 U/kg, *sc*) and sacrificed 15 min after heparin injection, during the morning of estrus phase. After decapitation, blood samples were collected and immediately centrifuged at 825 g at 4°C for 10 min to obtain plasma, which was kept at −20°C for future measurements of progesterone, estradiol, and testosterone by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) (Moysés et al. 2001).

Histological Analysis

Animals were perfused with sterile saline containing heparin (10 U/ml) via the left cardiac ventricle followed by infusion with 10% formaldehyde in phosphate-buffered saline (PBS-formalin). Ovaries were removed, fixed in PBS-formalin, pH 7.4, for 24–48 h at room temperature. After fixation, tissues were dehydrated in graded ethanol, cleared in xylol, and embedded in paraffin at 60°C and further sectioned into 5-μm slices. Sections were stained with hematoxylin and eosin (H&E). In each ovary, ovarian follicles and corpora lutea were counted in three sections per animal and expressed as number per unit area (mm^2), as described by Guerra et al. (2010). Follicles were classified according to Borgeest et al. (2002) and Talsness et al. (2005). Follicles were classified as preantral when containing two to four layers of granulosa cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte, and detachment from the basement membrane. Primordial and primary follicles were not counted. In each section, 5 different regions were analyzed, resulting in a total of 15 measurements per animal. For histological analysis, an image analysis system was used that consisted of a digital camera (Evolution, Media Cybernetics, Inc., Bethesda, MD) coupled to

a light microscope (Eclipse 400, Nikon). High-quality images (2048 × 1536 pixel buffer) were captured with Pro Plus 4.5.1 software (Media Cybernetics).

Cell Culture and Micronucleus Assay

Immortalized Chinese hamster epithelium ovary (CHO-K1; Kao and Puck 1968; Puck et al. 1958) cells obtained from Londrina State University (State University of Londrina, UEL, Brazil) were used to perform the antimutagenic assay. Culture flasks (25 cm^2) were used to seed monolayer cultures in DMEM/HAM F12 medium, supplemented with 10% bovine fetal serum (FBS) and 0.1% antibiotic–antimitotic solution. The flasks were maintained in a BOD incubator at 37°C. Under these conditions, the cell cycle is 12 h. After reaching 90% confluence, cells were incubated with DMEM in the absence of FBS for 12 h, and incubated with TBT (TBT_1: 2×10^{-3}; TBT_2: 2×10^{-2}; TBT_3: 2×10^{-1}ng/ml) for 3 h. The negative control was phosphate-buffered saline (PBS, pH 7.4) without TBT. Positive controls were mutagenic agent methyl methanesulfonate (MMS, 4×10^{-4} *M*) without TBT. Cytochalasin (3 μg/ml) was added in culture medium with CHO-K1 cells for 18 h to induce formation of binucleated cells. The micronucleus (MN) assays were conducted in triplicate. The fixer was removed until cell dilution for slide mounting was achieved, followed by staining with 5% Giemsa. One thousand cells per treatment were analyzed according to Malini et al. (2009).

Statistical Analysis

Data are reported as means ± SEM. Comparisons between groups were analyzed by Student's *t*-test (unpaired) and Tukey's test. Statistical significance was was considered when $p < .05$.

RESULTS

Prior to treatment with TBT, females demonstrated regular 4–5 d of estrous cycles in all experimental groups. Following TBT treatment, rats exhibited an abnormal pattern of estrous cyclicity. TBT exposure significantly decreased regularity of the estrous cycle (%) in four consecutive estrous cycles (Figure 1). In addition, progesterone levels significantly increased and 17β-estradiol decreased when compared to control (Table 1). Testorerone levels, however, remained without marked changes.

Experimental groups did not differ significantly as to body weight (data not show) and organ weight/body ratio for adrenal, liver, and uterus (Table 1). However, the ratio of ovary/body weight decreased significantly compared to control. The durations of the proestrus and diestrus phases of the reproductive cycle were significantly lower; however, metestrus length markedly rose, and despite this increase, there was an overall reduction in the cycle duration following TBT administration (Table 2). Further, the analyses of the vaginal smear showed a significant decrease in the number of epithelial cells during proestrus, and elevated number of cornified cells during metestrus after TBT treatment (Figure 2).

Ovarian tissue of control and TBT-treated exhibited morphological differences (Figure 4).

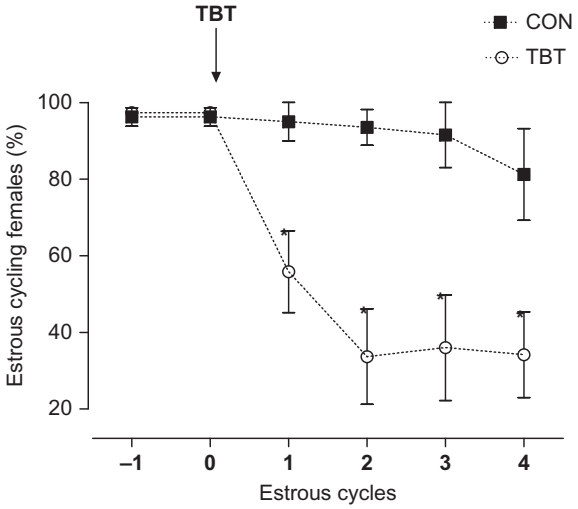

FIGURE 1. Percentage values of the estrous cyclicity in control female rats (CON) and tributyltin-treated (TBT) rats. Values are reported as the mean ± SEM. *$p < 0.05$ compared with the control group (unpaired Student's t test, n = 10 per group).

TABLE 1. Summary Data of the Plasma Concentration of Female Sexual Hormones and Liver, Ovary, Uterine, and Adrenal Weights in Female Rats Control and Treated With TBT

Groups	O.W. (mg/100g b.w.)	U.W. (mg/100 g b.w.)	L.W. (mg/100 g b.w.)	A.W. (mg/100g b.w.)	E_2 (pg/ml)	P_4 (ng/ml)	Testosterone (ng/dL)
CON	11.5 ± 1.0	51.6 ± 0.8	0.94 ± 0.02	0,09 ± 0.02	47.2 ± 7.0	4.0 ± 0.7	4.8 ± 0.8
TBT	8.7 ± 0.9*	51.3 ± 4.2	0.90 ± 0.04	0,10 ± 0.00	32,3 ± 4.3*	7.0 ± 1.2*	4.3 ± 0.2

Note. Values are reported as mean ± SEM; 17β-estradiol (E_2); progesterone (P_4); body weight (b.w.); liver weight (L.W.); ovary weight (O.W.); uterine weight (U.W.); adrenal weight (A.W.). Asterisk indicates significant at $p < .05$ compared with the control group (unpaired Student's *t*-test, $n = 10$ per group).

TABLE 2. Assessment of Estrous Cycle Length and Frequency of Each Phase Over a 16-d Period of Evaluation in Female Control and TBT-Treated Groups

Estrous cycling	CON	TBT
Frequency of proestrus(days)	1.1 ± 0.08	0.7 ± 0.12*
Frequency of estrus (days)	0.8 ± 0.07	0.8 ± 0.09
Frequency of rnetestrus (days)	0.9 ± 0.07	1.3 ± 0.10*
Frequency of diestrus (days)	1.0 ± 0.08	0.5 ± 0.12*
Estrous cycle length (days)	3.8 ± 0.11	3.2 ± 0.12*

Note. Values are expressed as mean ± SEM. Asterisk indicates significant at $p < .05$ compared with the control group (unpaired Student's *t*-test, $n = 10$ per group).

Control rats showed some antral follicles (Figure 4A), corpus luteum (Figure 4A and B), and structure of follicle (Figure 4C) with normal aspect. TBT-treated rats demonstrated atretic ovary, with presence of cystic follicles and corpus luteum (Figure 4D). Apoptotic cells were found in central area of corpus luteum (Figure 4E), granulosa cell layer (Figure 4F) and antral space (Figure 4G). In addition, treatment with TBT displayed a significant level of unbalance in some ovarian follicles, as evident from increased number of atretic follicles and corpora lutea (Table 3).

In vitro MN analysis in CHO-K1 cells showed a significant concentration-dependent mutagenic effect of TBT with TBT_1 at 2×10^{-3}ng/ml and TBT_2 at 2×10^{-2}ng/ml after 3 (Figure 3). With TBT_3 at 2×10^{-2}ng/ml, most CHO-K1 cells were in suspension and not viable (data not show).

DISCUSSION

This study showed that TBT, when administered in doses below what was proposed by the U.S. Environmental Protection Agency (EPA) (0.3 μg/kg; U.S. EPA 1997) to adult female rats with regular estrous cycle, showed decreases in ovarian weight and impaired balance of ovarian sex hormones. Further, this compound produced damage in estrous cyclicity, reduced the number of normal estrous cycles, increased the number of atretic follicles and corpora lutea, and elevated the number of MN in CHO-K1 cells.

Organotin compounds are chemicals composed of tin, directly bound to a number of organic groups (Swedenborg et al. 2009). Human exposure to OT occurs through fish and shellfish consumption (Appel 2004). TBT exposure was reported to alter the functions of a variety of endocrine tissues, such as the pituitary, endocrine pancreas, gonads, and thyroid glands in rats (Delgado Filho et al. 2010; 2011; Kishta et al. 2007). Triorganotins are endocrine disruptors with the potential to disturb hormonal regulation and the normal endocrine system, consequently affecting health and reproduction in animals and humans (Casals-Casas and Desvergne 2011).

Published observations on the effects of TBT on ovary weight are controversial. Female Wistar rats treated at postnatal day 23 and 33 with TPT (2 or 6 mg/kg/d) demonstrated increased ovarian weights (Grote et al. 2006). In contrast, chronic dietary exposure of rats to 50 mg bis(tri-*n*-butyltin) oxide (TBTO)/kg diet produced a decrease in ovarian weights (Wester et al. 1990). These findings are contradictory to results reported by Ema et al. (1999), who observed no significant difference in ovarian weights in pseudopregnant rats treated with 3.1, 4.7, or 6.3 mg triphenyltin

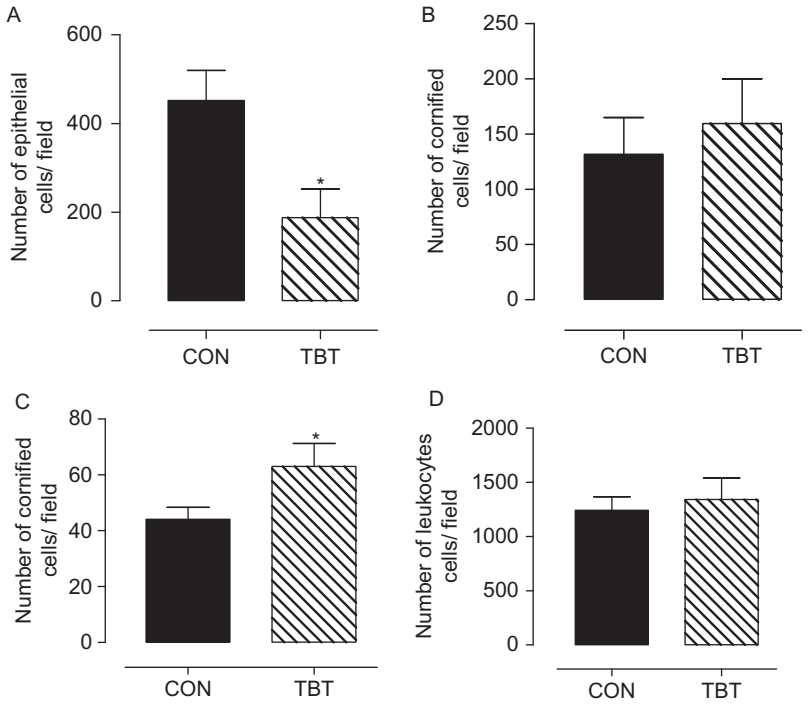

FIGURE 2. Cell number of vaginal smear from sexually mature female rats at different phases of reproductive cycle after treatment with TBT. (A) Proestrus Phase: number of epithelial cells in the proestrus phase in CON and TBT. (B) Estrus Phase: number of cornified cells in the estrus phase in CON and TBT. (C) Metestrus Phase: number of cornified cells in the metestrus phase in CON and TBT. (D) Diestrus Phase: number of leukocytes cells in the diestrus phase in CON and TBT. All the graphics showed CON and TBT groups as indicated on the abscissa and the columns indicate means ± SEM of 320 captured images (160 from control-group and 160 TBT-group) from each daily vaginal smear in the different experimental groups (n = 10 per group). *p < 0.05 compared with the control group (unpaired Student's t test).

TABLE 3. Ovarian Follicles and Corpora Lutea Counting per Unit Area (Number/mm^2) in Female Control and TBT-Treated Groups

Structures	CON	TBT
Pre-antral follicles	0.93 ± 0.11	1.10 ± 0.10
Antral follicles	0.90 ± 0.10	0.93 ± 0.11
Atretic follicles	0.75 ± 0.10	1.80 ± 0.20*
Corpora lutea	0.80 ± 0.09	1.10 ± 0.07*

Note. Values are expressed as mean ± SEM. Asterisk indicates significant at $p < .05$ compared with the control group (unpaired Student's *t*-test, $n = 4$ per group).

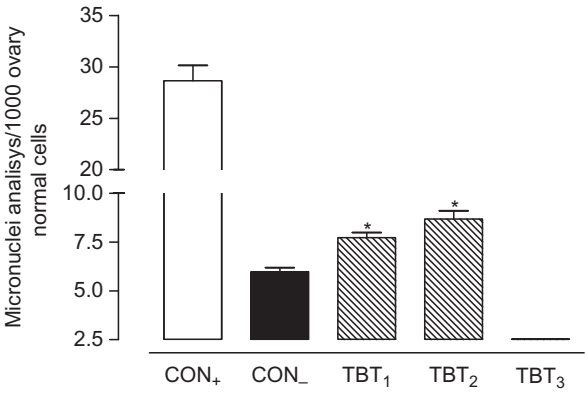

FIGURE 3. Baseline values of mutagenic parameters (micronucleus test) in CHO-K1 cells treated with different doses of TBT (TBT1: 2.0. 10-3; TBT2:2.0. 10-2; TBT3:2.0. 10-1ng/ml). All cells that were evaluated were dead in TBT3. CON-: PBS-treated without TBT. CON+: MMS-treated (mutagenic agent) without TBT. *p < 0.05 compared with CON- (Tukey's test, n = 5 per treatment). Values are reported as the mean ± SEM.

chloride (TPTCl)/kg. Our results indicate that female rats exposed to low doses of TBT may be more susceptible to the estrous cycle disturbances, reduced ovarian weight, and imbalance in ovarian sex hormones.

Based on biochemistry studies, the conversion of androgens to estrogens is catalyzed by aromatase activity in specific brain areas, gonads, adipose tissue, and several other tissues (Grote et al. 2006). It was suggested that OT increase androgen levels via inhibition of

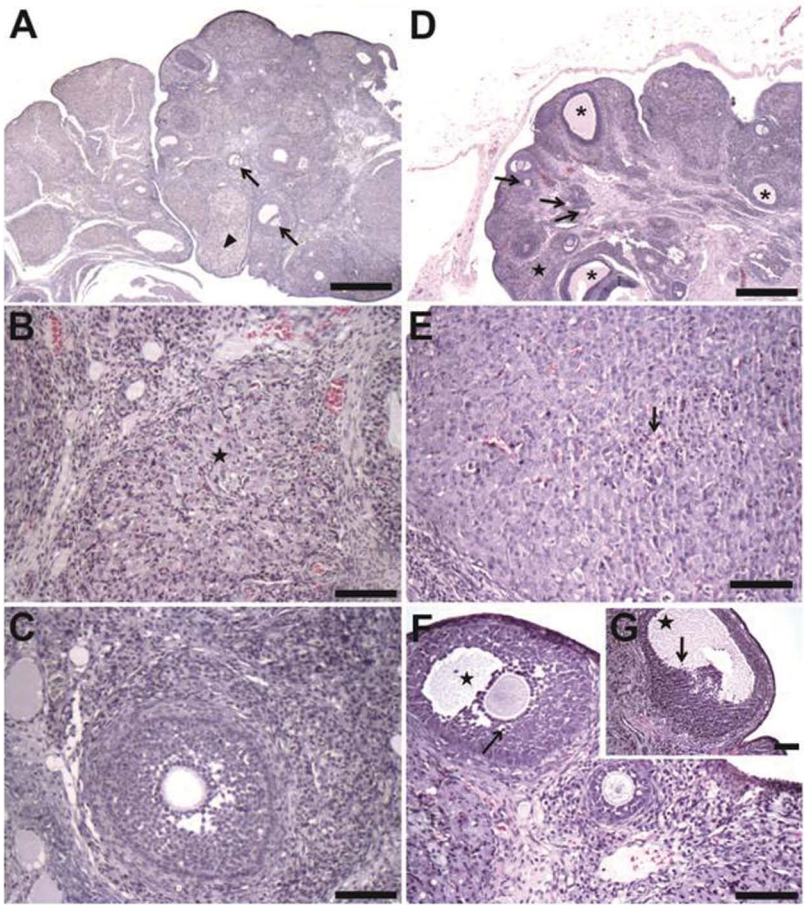

FIGURE 4. Photomicrography of HE-stained ovary sections from CON and TBT rats. (A) Low magnification photomicrography of CON ovary depicting some antral folicles (arrow) and corpus luteum (arrowhead, HE staining, bar: 400mm). (B) CON rat corpus luteum (star, HE staining, bar: 400mm). (C) CON Follicle with an organized structure (HE staining, bar: 200mm). (D) Atretic ovary in TBT (asterisks). Presence of cystic follicles (arrow) and corpus luteum (star) (HE staining, bar: 200mm). (E) TBT animals present corpus luteum with apoptotic cells (arrow) in the central area (HE staining, bar: 200mm). (F) Atretic antral follicle with intact oocyte (star). Some dead cells (arrow) in the granulosa cell layer in TBT (HE staining, bar: 200mm). (G) Atretic antral follicle without oocyte (star). Some granulosa cells of the atretic follicle were apoptotic (arrow) in the antral space in TBT (HE staining, bar: 200mm) (color figure available online).

aromatase activity in mollusks (Matthiessen and Gibbs, 1998; Oehlmann et al. 1996; Ronis and Mason 1996).

Saitoh et al. (2001) demonstrated that treatment with 20 ng/ml TBT or TPT for 7 d in the human granulosa-like tumor cell line induced a significant decrease in estradiol concentrations, which was consistent with the changes observed in the aromatase mRNA expression and activity. Others studies in vitro demonstrated that TBT is an inhibitor of human aromatase activity (Cooke et al 2002; Heidrich et al. 2001). In contrast, Nakanishi et al. (2002) noted a significant rise in aromatase activity in human placental choriocarcinoma cells after treatment with TBT and TPT. On the other hand, studies in vivo demonstrate that TBT decreased ovarian aromatase activity on postnatal day 53 female Wistar rats after exposure to 6 mg TPT/kg (Grote et al. 2006). Some findings indicate that OT are also potent agonists for the nuclear receptors retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor PPARγ (Grün and Blumberg 2006). Both RXR and PPAR-g selective ligands suppress aromatase gene expression in the ovary (Fan et al. 2005; Mu et al. 2001) via nuclear factor-kB. (Fan et al. 2005).

Sex steroids influence the growth, function, and differentiation of female reproduction organs and make them susceptible to endocrine disruption (Guerra et al. 2010). During the estrous cycle, fluctuating levels of estrogen and progesterone elicit profound effects on epithelial proliferation and cytodifferentiation (Boutin and Cunha 1997). For example, estradiol levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus. Progesterone secretion also rises during metestrus and diestrus with a subsequent fall (Sportnitz et al. 1999). Decreased duration and proportion among three cell types of reproductive cycle were found. There were proportional differences among cell types in estrous cycle between control and TBT, suggesting that proportional variation between phases is dependent on the levels of sex hormones. In addition, the reduced serum estradiol and elevated serum progesterone levels suggested some ovarian dysfunction, because changes in plasma female steroids indicate impaired ovarian function and may be produced by direct effect of TPT on the ovary or by disruption of the hypothalamic–pituitary feedback mechanisms (Grote et al. 2006).

In mammals, different levels of TBT change the functions of a variety of endocrine tissues such as the pituitary, pancreas, gonads, and thyroid glands (Oberdorster et al. 1998; Vos et al. 2000; Wada et al. 1982). Others studies showed that TBT induced apoptosis in hepatocytes (Reader et al. 1999) in human Hut-78 and Jurkat T-lymphocyte cells (Stridh et al. 1999). An imbalance between development of ovarian follicles with cystic follicles, apoptotic cells in corpus luteum, and granulosa cells was noted, as well as a rise in number of atretic follicles after TBT treatment. Similarly, Kistha et al. (2007) demonstrated an increased number of degenerating and apoptotic germ cells in fetal ovaries of TBT-treated rats.

The corpora lutea play a central role in the regulation of the estrous cycle and in maintenance of pregnancy. This function is carried out largely by progesterone, which is the main steroid synthesized by this transient endocrine gland (Stocco et al. 2007). Reduction in the formation of corpora lutea may be associated with changes in the ovarian weight (U.S. EPA 1996). Ema and Miyawaki (2002) demonstrated that the ovarian weight and number of corpora lutea of rats on d 4 of pseudopregnany treated with DPT are similar to control; however there was increased ovarian weight at 24.8 mg/kg DPT on d 9 of pseudopregnancy and the number of corpora lutea did not change. Our findings showed an increase in the formation of corpora lutea with decrease in ovarian weight and increase of progesterone levels after TBT treatment.

Peluso (2003) suggested that the granolusa-cell progesterone synthesis and caspase activation are intimately interconnected with each other. In most cell types, activation of caspase-3 results in apoptosis, for example, in rat preovulatory granulosa cells (Yuan et al. 2012). Cytochrome P-450scc enzyme catalyzes progesterone production and leads to an increase in reactive oxygen species (ROS) formation (Yacobi et al. 2007), which exert a pro-apoptotic capase-3-stimulating effect (Nowicki et al. 2007). Progesterone production rise was associated with signs of apoptosis in bovine granulosa cells (Hummitzsch et al. 2009). Endocrine disruptor chemicals, such as TBT (Grote et al. 2006), have been implicated in numerous physiological processes affecting normal reproductive health (McLachlan 2001).

TPT and other OT inhibit aromatase enzyme activity in rat (Grote et al. 2006) and mollusks (Nakanishi et al. 2008). OT induce apoptosis or necrosis in mammalian cells (Nakanishi et al. 2002; 2006; Saitoh et al. 2001). For example, cell proliferation was suppressed after exposure to 200 ng/ml of TBT for 48 h in the human granulosa-like tumor cell line (Saitoh et al. 2001). In addition, exposure of fish cell embryos in the gastrula period with TBT (10 ng/L) for 144 h stimulated cell apoptosis (Zhang et al. 2011). Triorganotin was also reported to induce apoptosis in mammalian HL-60 promyelocytic cells, by means of a cascade of events that include (i) increase of intracellular Ca^{2+}, (ii) alteration of actin polymerization, and (iii) induction of DNA degradation. Furthermore, our in vitro experiments

using CHO-K1 cells showed differences in MN numbers between control and TBT groups, suggesting that increase of MN in these cells is dependent on TBT exposure concentration. Other data showed that TBT induced activation of caspases and consequent apoptosis of invertebrate hemocytes (Cima and Ballarin 1999) and rat pheochromocytoma PC12 cells (Nakatsu et al. 2007).

Evidence indicates that under some experimental conditions low doses of TBT impair the estrous cyclicity, at least in part, by imbalance in ovarian sex hormones, alterations in number of cells from vaginal smears, ovarian weight, and number of atretic follicles and corpora lutea. The characterization of these mechanisms may lead to a better understanding of effects of TBT in women and perhaps to better forms of therapy.

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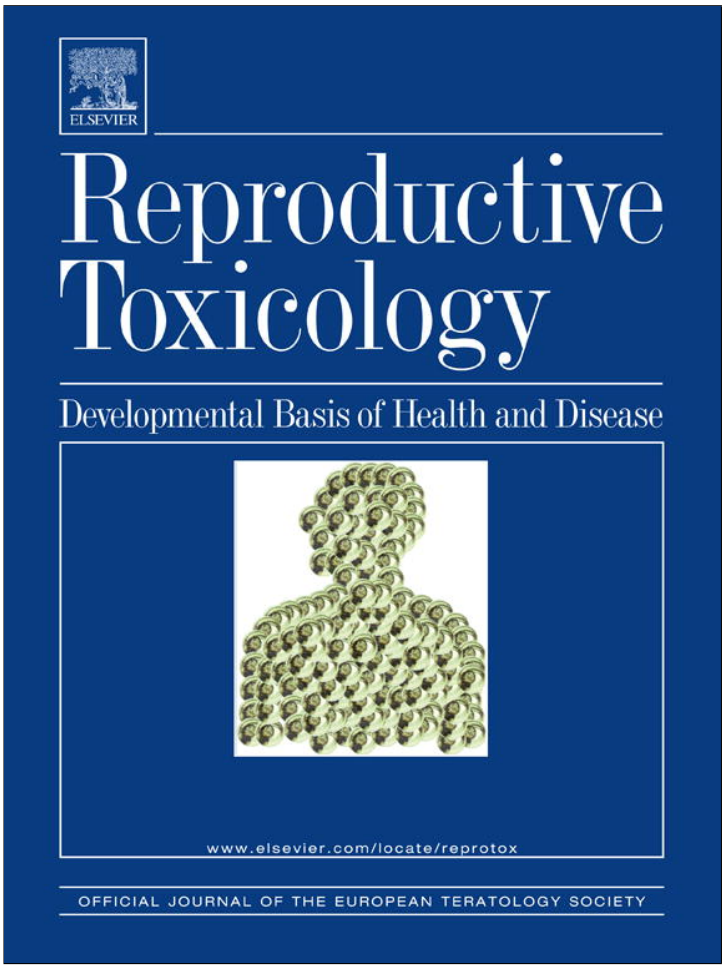

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Organotins: A review of their reproductive toxicity, biochemistry, and environmental fate

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ABSTRACT

The review purposes are to (1) evaluate the experimental evidence for adverse effects on reproduction and metabolism and (2) identify the current knowledge of analytical procedures, biochemistry and environmental aspects relating to organotins. Organotins are pollutants that are used as biocides in antifouling paints. They produce endocrine-disrupting effects in mollusks, such as imposex. In rodents, organotin exposure induces developmental and reproductive toxicity as well as alteration of metabolic homeostasis through its action as an obesogen. The adverse effects that appear in rodents have raised concerns about organotins' potential health risk to humans in relation to organotin exposure. At present, triorganotin, such as tributyltin, have been demonstrated to produce imposex, and mammalian reproductive and metabolic toxicity. For most mammals, triorganotin exposure predominantly occurs through the ingestion, and this compound can cross the placenta. With these risks in mind, it is important to improve our knowledge of organotins' effects on environmental health.

1. Introduction

Organotins (OTs) belong to an organometallic class of pollutants. They are composed of an atom of tin that is covalently bonded to one or more organic chains [1] and another functional group, such as chloride, oxide, or hydroxide, which are represented by methyl, butyl, octyl, and phenyltin groups. The existence of OTs has been known since 1853, but they did not become important for industrial use until the 1940s. Since then, they have come into extensive use in several industrial sectors, mainly as biocides in antifouling boat paints [2]. Antifouling paints are used to reduce encrustations by barnacles, algae, mussels, and other marine invertebrates [3]. Antifouling solutions are based on two main triorganotins, tributyltin (TBT) and triphenyltin (TPT), which are the most toxic OTs.

OTs commonly break down in the environment. For instance, TBT degrades to dibutyltin and monobutyltin, and TPT degrades to diphenyltin and monophenyltin. The final degradation product is inorganic tin (Sn^{4+}), which is stable. Because of their organic chains, OTs are hydrophobic; this feature depends on the alkyl/aryl bond of the tin atom (in other words, the number of groups and the length of the organic chain). These compounds have low solubility in water, and their solubility depends on their pH, ionic strength, and temperature. In aqueous solutions, they can appear as neutral, ionically paired complexes and cations [4]. There is a relationship between the chemical form of OTs and their transport properties, bioavailability, and toxicity in the environment [5]. These properties can also be influenced by the organic matter content of the environment or the specific tissue in question [6,7].

The toxicity of OTs is controversial; their level of toxicity may be related to their concentration, exposure time, bioavailability, and biota sensitivity as well as the presence of various compounds in the environment. OTs are a diverse group of widely distributed environmental pollutants [8] that have been implicated as endocrine disruptors [9]. The increase in OTs toxicity may be related to their insolubility in water because their hydrophobicity is the main chemical characteristic that is responsible for their

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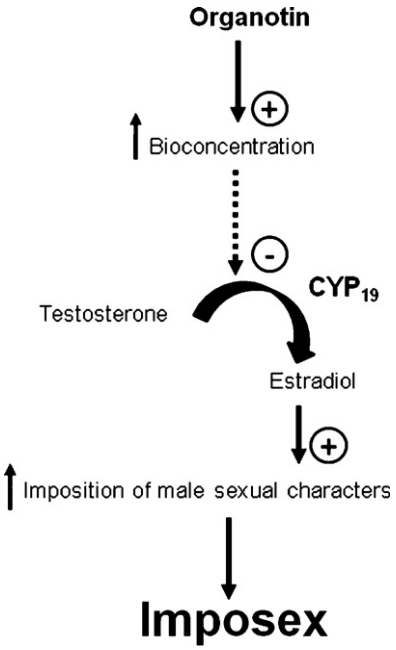

Fig. 1. Diagram illustrating the down-regulation of effective aromatase activity (CYP 19) mediated by organotin in mollusks, inducing imposex. The dashed arrow and the continuous arrows indicate an inhibitory (−) and a stimulatory (+) effect, respectively.

bioconcentration [10]. Usually, maximum toxicological activity is found when organisms are exposed to triorganotins, such as TBT [1].

OTs are markedly toxic to mollusks and can produce endocrine-disrupting effects. For instance, TBT and TPT can induce imposex, or the imposition of male sex characteristics in female snails [11,12]. The mechanism by which these OTs cause imposex is unclear, but OTs are known to induce the inhibition of one aromatase, a cytochrome P450 that converts testosterone into estradiol [12] (Fig. 1). This process occurs in a dose-dependent manner [13] making it useful as a biomarker for different contamination levels [14].

OTs are also suspected to cause endocrine-disrupting effects in mammals, humans [15] and rodents [16,17], in part as a consequence of the consumption of contaminated seafood. Human exposure may result from dietary sources, such as seafood, or through contaminated drinking water [18]. *In vitro* exposure to TBT or TPT in human choriocarcinoma cell lines decreases DNA and protein synthesis [19]. TPT inhibits human aromatase [20] and other steroidogenic enzymes, affecting sexual development in rodents [16,21,22]. Therefore, OTs have many complex effects on the endocrine systems of both genders that can induce morphological changes in the target organs.

TBT is also a potent agonistic ligand of vertebrate nuclear receptors, retinoid X receptors (RXR) and peroxisome proliferator-activated receptor-gamma (PPARγ). The physiological consequences of receptor activation have been demonstrated in experimental models of adipogenesis [8]. Thus, TBT alters metabolic and lipid homeostasis parameters, induces the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo* [8,23].

2. Analytical procedures and biochemistry

A variety of analytical techniques have been developed to study the speciation of OTs, mainly in sediments and biota [24]. The choice of a suitable solvent is the first step and is based on the solvent's ability to extract the various OTs that are in the matrix, which in turn depends on the nature of intermolecular forces, the geometric arrangement, and the balance between the polar and non-polar characteristics of the OTs to be extracted [5].

2.1. Extraction

Because OTs are bonded to the sediment surface, complete matrix dissolution is not required. More than half of the existing extraction procedures use a combination of an organic solvent of low to medium polarity with an acid [25]. Hexane, toluene, or dichloromethane are the solvents that are most often used in combination with acetic or hydrochloric acids. Liquid–liquid extraction and Soxhlet extraction are the classic methods, although they are used less often than the ultrasonic radiation technique that is currently in popular used for OTs extraction [26].

2.2. Derivatization

The technique most often used for derivatization is the Grignard reaction [5,27]. Ethylation and pentylation are usually employed because they allow for the determination of methyl, propyl, butyl, and phenyltin species [5]. Alkylation reactions with hydride generation reactions have been used to produce volatile derivatives. Sodium tetrahydroborate ($NaBH_4$) and sodium tetraethylborate are frequently used in this process [28]. Generally, the reduction is performed at a pH below the pK_a of the species of interest [29]. This method provides good sensitivity for aqueous samples. However, for complex samples (from sediments and biota), it presents a number of disadvantages, such as the limited number of compounds that can be determined and the instability of $NaBH_4$ [25].

2.3. Cleanup

A cleaning procedure is needed to eliminate matrix components and improve analysis reliability. OTs are particularly sensitive to interferents, and extract purity is a concern [5]. Sulfur (in anoxic sediments) is co-extracted and alkylated during Grignard reagent derivatization, yielding mono-, di-, and trisulfide dialkylated derivatives. If these derivatives are not removed, they can interfere with the chromatographic analysis of OTs as a result of their coincident retention time (co-elution). Silica gel, alumina, and florisil (these are preferred for biotic matrices) are the most common adsorbents used during cleanup [25].

2.4. Separation procedures

Most of the analytical methods that were developed to quantify OTs require hyphenated techniques, which are detection techniques with a specific detector that is suitable and a quantifying technique for a specific element. About two-thirds of the techniques used in OT analysis are based on gas chromatography (GC) [5]. One advantage of GC is the use of internal standards and surrogates to verify some of the analytical steps, such as quantification and recovery. The main disadvantage of GC is the requirement for volatile derivatives [25]. Mono-, di-, and triorganotin compounds are not sufficiently volatile, and a derivatization step is therefore needed. However, many techniques that are based on high performance liquid chromatography (HPLC) have been developed for OTs analysis [30–33], but interfacing HPLC with detection systems can be challenging, and the number of compounds that can be analyzed in a single run is limited in comparison to GC [34].

2.5. Detection

There are a number of available methods that use some form of GC. Flame ionization detectors, electron capture detectors, atomic

absorption spectrometry, flame photometric detectors (FPD), atomic emission detectors and mass spectrometry are used most often [35,36]. The widely used FPD has many advantages because of its availability and its sensitivity to and high selectivity for OTs. Under particular conditions and in a hydrogen-rich flame, OTs are converted to SnH species, which give off intense emissions in the 360–550 nm (wide blue peak) and 600–640 nm (narrow red peak) regions [37]. The emitted light is collected by a photomultiplier cell and subsequently passed through a filter with a specific wavelength to produce a detector signal, which relates to the mass of the analyte [38]. The red emission at 610 nm is preferred due to its high sensitivity, while the blue emission at 390 nm is avoided due to the co-elution of organotin with sulfur compounds. The pulsed flame photometric detector (PFPD) is recommended for OT analysis because it allows for a distinction between the background (using chemiluminescent gaseous products and OH radicals that form as a consequence of the hydrogen-rich flame, with a maximum at 436 nm) and the analyte emissions and improves sensitivity. Moreover, analyte emissions can be increased by changing the choice of the flammable gas mixture [39,40]. Numerous applications of this procedure can be found in the literature [25,41,42].

2.6. Biochemistry of endocrine-disrupting effects

The production of sex steroid hormones from cholesterol molecule to be trafficked between the mitochondria and smooth endoplasmic reticulum and involves many enzymatic steps [43]. Most of these pathways use cytochrome P450 enzymes that contain heme. Some OTs are known to be encoding-disrupting chemicals that affect steroid hormone biosynthesis in mollusks [44,45], rodents [17] or different cellular lines [46,47].

Butyltins are capable of inducing a concentration-dependent inhibition of human cytochrome P450 aromatase activity in microsomal human placenta cells with concentrations ranging from 0 to 200 mM after 15 min of incubation [48] or in insect cells (BTI-TN-5B1-4) that have been transfected with human CYP 19 cDNA and human P450 reductase [46]. However, this inhibition took longer at the effective concentrations (20 ng/ml for 48 h) for the inhibition of aromatase in human granulose-like tumor cell (KGN) [47]. Exposure to TBT or TPT concentrations higher than 300 nM markedly decreases DNA and protein synthesis in the human choriocarcinoma cell lines Jar, JEG-3 and BeWo [19,49]. Concentrations under 1 μM of either OTs do not significantly affect aromatase activity in human choriocarcinoma cells [19]. In addition to aromatase, TBT inhibits the catalytic activity of human 5α-reductase I and II (5α-R I and II) [50], rat 3β-hydroxysteroid dehydrogenase (3β-HSD) [51] and pig 17β-hydroxysteroid dehydrogenase I (17β-HSD I) at levels above 1 μM [52]. TPT inhibits the catalytic activity of human aromatase, 5α-R II, and 17β-HSD I and III [20]. These observations suggest that these OTs do not specifically inhibit the catalytic activity of aromatase at low levels. However, TBT interferes with the binding of androstenedione to aromatase. When microsomal human placenta preparations are incubated with 1.6 mM TBT and 0.1 mM [1b-3H] androstenedione (the aromatase substrate) over a 30 min time period, the TBT-induced decrease in aromatase activity remains constant when the reactions are carried out over various incubation times, indicating a reversible inhibitory mechanism [48]. We should also distinguish between nonspecific OTs cell toxicity and the specific inhibition of steroidogenic enzymes from *in vitro* and *in vivo* studies.

Based on endocrine studies, gonadal steroid receptors and steroidogenic enzymes for sex steroid hormones have not yet been identified in gastropods and it remains unclear if sex steroid hormones are critical for sexual maturation in these animals. Furthermore, estrogen and androgen receptor homologs have not been found in invertebrates [53] and the composition of nuclear receptor family members is very different between vertebrates and invertebrates [54]. Therefore, there is some doubt as to whether OTs might function as agonists for nuclear receptors [55] and/or inhibitors of enzymes that metabolize androgens in gastropods, leading us to suspect that OTs may affect other target molecules. Thus, the extension of OTs effects on invertebrates or mammals and/or their abnormal effects on hormonal modulation to their reproductive and metabolic functions is still not thoroughly known, even after they have been the focus of numerous investigations. This lack of knowledge should be taken into account when investigating the ubiquity of OTs in coastal ecosystems or seafood.

3. Environmental aspects

TBT, TPT and their degradation products are present in all compartments of the coastal environment [56]. The degradation of TBT is considered to be a fast process in the water column, with a half-life on the order of days [57]. However, the half-life of OTs can be very long in sediments, on the order of years [56]. The bioaccumulation of OTs in the food chain introduces the potential for contaminant transfer by contact with drinking water and foods [18,58,59]. Their toxic effects may cover one or more levels of biological organization within an ecosystem [60,61].

The negative effects of TBT have been known since the 1980s when the first records of imposex were reported in *Nucella lapillus* (Linnaeus, 1758) [62] and *Ilyanassa obsoleta* (Say, 1822) [63] on British and French seashores [64]. Low doses of TBT affected larval development and inhibited calcification of oysters, which led to decreased oyster production [64,65]. TBT is considered to be a chronic contaminant and has the following effects: levels > 1 ng/l limit cell division in phytoplankton and zooplankton reproduction; levels > 2 ng/l are responsible for shell calcification anomalies in the oyster *Crassostrea gigas*; levels > 20 ng/l impair bivalve mollusk reproduction; levels at 1–10 mg/l alter fish reproduction; levels of 1–1000 mg/l induce disturbed fish behavior; and levels of approximately 500 mg/l showed changed crustacean exuviations [65,66]. Additionally, OTs may accumulate in birds and sea mammals [67] and can limit growth rates and photosynthesis in algae [68].

In mollusks, TBT acts by impairing the reproductive function and can induce imposex, intersex and the formation of ovo-testis. These phenomena are widely distributed in several countries and occur in approximately 268 species of gastropods [69]. The possible mechanisms for OT-induced imposex include: (1) increased testosterone levels by aromatase [9,45]; (2) acyl CoA-steroid acyltransferase inhibition [70]; and (3) diminished androgen excretion of sulfate conjugates [71]; (4) another hypothesis, although less accepted, is a neurotoxic effect of TBT that could induce imposex *via* the abnormal release of APGWamide [12]; and (5) activation of RXR [72].

Imposex intensity is a measurement of the extent of female masculinization and can be described in many different ways. For instance, the rate of the number of affected females can be expressed as a percentage of imposex incidences among the females in each population [% imposex (%I)]. A comparison of the lengths of female penises with those of males, known as the relative penis length index (RPLI) and relative penis size index (RPSI), is also a measurement of imposex intensity. However, these indexes are influenced by seasonality. The most widely used index is the vas deferens sequence index (VDSI). In highly polluted areas, the male (M) to female (F) ratio (M: F) can be useful because in some species, female mortality as a consequence of OTs exposure [69,73].

The VDSI is the most significant biological parameter because it estimates the extent of the reduction in reproductive females [13] and faithfully reflects TBT-induced vas deferens development.

Oehlmann et al. [74] developed a VDSI index that has seven stages (0–6). The first stage refers to normal females, with no signs of masculinization, and the sixth stage included sterile females with the vulva blocked by the growth of vas deferens tissues and the presence of aborted egg capsules in the lumen of the gland capsule. Accumulation of these egg capsules can lead to gland rupture and the subsequent death of the female. The rate of sterile females has been used as an indicator of declining populations and is common in areas with intense naval activities [73]. The %I is used especially in places with an apparent decline in environmental TBT levels [69]. The M:F ratio is used to evaluate deviations from the expected ratio between male and female gastropods. Populations composed predominantly or only of males indicate sites that have been severely impacted by OTs pollution [69], and include higher female mortality and extinction [73]. The RPSI or RPLI are commonly used in comparisons between measures of the length and average volume of the penises of females and males [73]. However, it is important to consider the existence of seasonal variations in penis size [13].

Intersex mainly affects mesogastropods, such as sea snails. This syndrome was previously observed in *Littorina littorea* [75]. It consists of the gradual replacement of the palial oviduct with a female prostate. There are four reported indexes: (1) intersex incidence, (2) % of sterile females, (3) intersex index (ISI) and (4) mean female prostate length (FPrL). Intersex incidence and percentage of sterile individuals are equivalent to %I and % of sterile females for imposex-affected species. The most widely used parameter is the ISI, which consists of five stages [75,76]. These vary from normal females (stage 0) to females with a prostate gland, penis and sperm groove (stage 4). It should be highlighted that intersex manifests at higher concentrations of TBT (15 ng Sn/l) [76], while imposex occurs at a level of approximately 1 ng Sn/l [77].

The third TBT-induced syndrome is ovo-testis formation, which was observed in the archaeogastropod *Haliotis madaka* [77]. In this species, there is a masculinization of the ovaries in which gonadal tissue is presented with the development of testicular tissue. Horiguchi et al. [77] suggest that this phenomenon is similar to the imposex development that occurs in meso- and neogastropods that is induced by OTs, although in the ovo-testis development, the formation of a penis does not occur.

Due to the toxicity of triorganotins, such as TBT, the International Maritime Organization (IMO) proposed a convention for the control of dangerous antifouling systems on ships in 2001; the convention was implemented in September 2008, and it banned the use of TBT-based paints on ships [78]. At present, 74% of the total worldwide commercial fleet is registered in countries that are signatories of the AFS convention and thus are expected not to carry OTs on their hulls. However, beyond its regular use in agriculture and other industries, it is possible that TBT is still used in antifouling paints in some parts of the world in countries that are not included in the AFS convention [79], especially in developing countries, such as Brazil [36], Ecuador [80] and Argentina [81].

In this scenario, contrasting reports on reproductive disorders caused by OTs would be expected. In those countries that have had enforced regulations on OT boat applications since the early 1980s, imposex has shown a marked decline [82,83]. This decrease is an indication of two conditions: (1) the reduction of OT pollution and (2) the antifouling paints used for local boats have been changed to tin-free formulations (third generation paints). In many instances, these paints are still not biocide-free and may also be toxic and have a strong sinergical interactions, which presents another pattern of toxicity for local communities [84,85]. However, there are still many countries, mainly among the developing countries, that do not enforce the AFS convention; they demonstrate a very different environmental state. In these instances, OT pollution is still present [81,86–89]. These areas are thus expected to present a mixed scenario of OTs and new antifouling paints being used at the same time, causing environmental and health consequences that are very difficult to evaluate [85]. Therefore, a health risk for heavy seafood consumers still remains [15,61,90,91].

4. Reproductive toxicology in the mammalian gonadal system

Sexual differentiation is a sequential process that begins with the establishment of chromosomal sex at fertilization, followed by the development of gonadal sex organs, and culminating in the development of secondary characteristics in both genders [92]. The endocrine system deeply affects reproductive morphophysiology, most likely from the influence of specific genes, with the actions of gonadal steroids and of the endocrine/paracrine pathways on the gonadal system [93]. Studies have suggested that the potential toxicity of OTs to mammals (specifically humans and rodents) is endocrinopathic, as well as potentially teratogenic and toxic to reproduction and development [55] in both genders [17,21].

4.1. Male reproductive system

TBT was reported to induce adverse reproductive effects in male rats [17,21,94–101]. In one study, two generations of male rats were treated with 5, 25, and 125 ppm of oral tributyltin chloride (TBTCl). As a result, their testis, epididymis, ventral prostates and body weights decreased, mainly in the 125 ppm-treated individuals [71]. The 125 ppm-exposed rats demonstrated a vacuolization of their seminiferous epithelium, spermatid retention, and delayed spermiation [21]. Effects on the F2 generation were greater in comparison with the F1 rats [21]. Other studies have shown a decrease in the weight of the seminal vesicles [99] and in the weights of all male reproductive organs, except the testis at a dose of 15 mg TBT/kg for 30 days beginning at postnatal day (PND) 23 [16]. However, it is also important to take notice of TBT's capacity to induce acute damage because a single dose of TBT caused a delay to lumen formation in the seminiferous tubules and increased the numbers of apoptotic germ cells inside the rat tubules [97]. In addition, TBTCl exposure (at 20 mg/kg) in male Sprague–Dawley rats at PND 35–44 caused an increase in detached debris and sloughed cells in the tubules of the epididymis, and the seminal vesicles were narrowed and filled with epithelial cells [98]. Although sperm motility and morphology were not affected by TBTCl treatment (at 5, 25 and 125 ppm), the homogenization-resistant spermatid count was reduced to approximately 80% of the control in rats treated with 125 ppm of TBTCl in both the F1 and F2 generations [21].

Investigations have shown that TBT exposure could reduce serum testosterone levels [16,97,100,101], and others have shown that aromatase inhibition induces a "hyperandrogenic" state in male mammals [102,103]. The adverse effects of OTs exposure depend upon the dose, timing and development period of the animal. However, atrophy of the ventral prostate was revealed in rats that ate a TBTCl diet, which is seemingly contrary to a "hyperandrogenic" state. Moreover, prostatic atrophy caused by aromatase inhibition has been frequently reported [104,105]. The explanation for this phenomenon lies in the role of estrogen in stimulating the expression of the androgen receptor [106]. As a result, an estrogen receptor antagonist or low serum estrogen levels could also cause prostate atrophy [107]. Additionally, testosterone aromatization disorders have been linked to serious spermatogenesis damage in mice and monkeys [102,103].

OTs can cross the placental barrier and bioaccumulate in large quantities in the placenta and fetal tissues [9,108]. *In utero* exposure to OTs [94,96] alters the development pattern of pre- and postnatal rat offspring [94,96]. Pregnant rats that receive TBTCl orally from days 0 to 19 or from days 8 to 19 of gestation develop lipid droplets

in the cytoplasm of their Sertoli cells and gonocytes, with large intercellular spaces and abnormally dilated endoplasmic reticulum in both cell types (as shown by electron microscopy). Additionally, the numbers of gonocytes, Sertoli and Leydig cells are reduced after TBTCl treatment (at 10 and 20 mg/kg) [96]. Male offspring exposed to 2 mg TPTCl/kg *in utero* and during lactation (from gestation day 6 until weaning on PND 21) or from gestation day (GD) 6 until termination show a postnatal development that includes a decrease in body weight gain, reproductive organ weight and testosterone levels, as well a delay in the age of preputial separation [109]. In contrast, TBTCl exposure from GD 0 to 19 or 8–19 does not result in external malformations in rats, nor is there a change in sex ratios. However, exposure to 0.25, 2.5, or 10 mg/kg TBTCl from GD 0 to 19 results in an increase in the anogenital distances in male fetuses. However, the exposures that occurred from GD 8 to 19 have no effect. Additionally, there is a dramatic increase in the incidence of low birth weight fetuses after exposure to 20 mg/kg TBTCl [94].

In vitro analysis has demonstrated that TBTCl and TPTCl (triphenyltin chloride) (at exposures of 0–80 nmol/l for 24 to approximately 96 h) enhances the occurrence of rat Leydig cell (LC-540) apoptosis (*via* DNA ladder formation) in a time- and dose-dependent manner [97], which is most likely mediated by raised cytoplasmic Ca^{2+} levels and mitochondrial disorders. In addition, TBTCl reduces the testosterone production of rat Leydig cells.

These conflicting and adverse results in mammalian male reproductive systems indicate the complex toxic interactions among the different cellular pathways that are affected by OTs and may be dose-, species- and timing-dependent. For instance, an imbalance in sexual hormones, an alteration in the role of sexual enzymes, impaired cellular homeostasis and increased cellular apoptosis are all physiopathological changes related to OTs exposure.

4.2. Female reproductive system

A large body of evidence indicates that exposure to OTs can cause reproductive disruption in the female mammal reproductive systems [17,19,21,22,110–113]. TPTCl exposure of 3.1, 4.7, or 6.3 mg/kg by gastric intubation on GD 0–3 or at 6.3, 12.5, or 25.0 mg/kg on GD 4–6 animals prevents fetal implantation in a dose-dependent manner. The pregnancy rate is reduced after TPTCl is applied on GD 0–3 at 4.7 and 6.3 mg/kg and on GD 4–6 at 12.5 and 25.0 mg/kg. TPTCl treatment during early pregnancy causes implantation failure and decreased uterine weight and serum progesterone levels, but ovarian weight, the number of corpora lutea and estrogen levels remain at average levels [112]. Following postnatal analysis, TPTCl exposure increases the ovarian weight of female rats and estradiol levels (6 mg TPTCl/kg) on PND 53, but the ovarian weight is not changed on when exposure occurs on PND 33 [17]. Podratz el al. [114] showed that TBTCl (at 100 ng/kg for 15 days) induced impairment of rat estrous cycle regulation, ovary morphology and follicle development with concurrent decreases in estradiol levels. In female rats, *in utero* exposure to TBTCl (at 20 mg/kg) leads to a decrease in maternal weight gain and fetal weight, induces pre- and post-implantation losses [94,113], increased in pregnancy failure by 84.6% after 16.3 mg/kg [113], increases rat fetal toxicity after 25 mg/kg of tri-n-butyltin chloride treatment [115], alters the anogenital distance of female pups on PND 1 [22], causes precocious completion of the vaginal opening in postnatal females [109], reduces the number of germ cells by approximately 45% and induces morphofunctional changes in the ovaries of fetal female rats [96]. In addition, dibutyltin (DBT) dichloride (DBTCl, 0, 2.5 or 3.8 mg/kg) treatment of pregnant monkeys once daily by nasogastric intubation on days 20–50 of pregnancy (which is the period of organogenesis) decreases maternal body weight at 3.8 mg/kg, but there are no changes in the developmental parameters in surviving fetuses, including fetal body weight, crown-rump length, tail length, or placental weight [116,117].

In an *in vitro* human choriocarcinoma cell line Jar exposed to TBTCl and TPTCl (0, 1, 10, and 100 nM for 48 h), an increase in mRNA and catalytic activity for 17β-HSD I has been observed. TBTCl and TPTCl treatments of 1 nM and 10 mM, respectively, abolish these effects because of cell extinction [49] that occurs in a dose-dependent fashion following exposure to nontoxic concentration ranges [55]. However, human granulosa-like tumor cell line KGN exposure to TBT and TPT for 72 h at 20 ng/ml (~6 nM) suppresses both aromatase activity and aromatase gene expression by 30% [47], which is nearly pharmacologically equivalent to the doses that reportedly induce imposex in female gastropods [118].

The conversion of androgens to estrogens is catalyzed by aromatase in specific brain areas, gonads, adipose tissue and other tissues. It has been suggested that OTs increase androgen levels *via* the inhibition of aromatase activity in mollusks [44,119], a theory that was supported by results of *in vitro* studies in which TBT [48] and TBTCl [46] caused inhibition of mammalian aromatase activity [47,120]. Despite TPTCl-related decreases of *in vivo* ovarian aromatase activity and increases in estradiol levels on PND 53, the same effect is not observed at PND 33 in female rats [17]. This difference could be caused by enhanced feedback mechanisms along the hypothalamic-pituitary-gonadal axis. Although results from several *in vitro* studies have provided evidence for the aromatase inhibition hypothesis in mammals [46–48,120], there are no studies that confirm these results *in vivo*. Additionally, it has been shown that other key enzymes that catalyze steroid hormone synthesis, such as 5-reductase I and II [50] or 17β-HSD I [49,120], are inhibited *in vitro* by TBTCl. In this regard, female rats that are treated with TBTCl *in utero* have increased apoptosis in the fetal ovary with degenerating germ cells [96]. Therefore, this discrepancy may be due to the tissue-specific expression of aromatase as altered by dose, period and time of exposure.

In addition to the adverse effects of organotins on steroidogenesis, TBT and dibutyltin dibromide (DBTBr) have also been found in large quantities in rat placenta and fetal tissues, showing that organotins cross the placenta and accumulate in fetuses [9,94,108]. In one study, pregnant rats were orally exposed to TBTCl from GD 8 until GD 20 at concentrations of 0, 0.25, 2.5 and 10 mg/kg/day. Milk samples were then collected from the dam at PND 6 and PND 12. The level of TBTCl and DBTBr in the dam's blood was raised after being treated with 2.5 and 10 mg/kg/day, and the levels in the placenta were approximately 5-fold higher than the levels in the dam's blood. The concentrations in the whole fetuses were approximately 50% lower than those in the placenta at GD 20 (there were also increases of TBT and DBTBr in the livers and brains of the fetuses) while the levels of TBT and DBTBr in the dam's milk declined between PND 6 and PND 12 [108]. Therefore, fetuses are at great risk of adverse effects from TBT *in utero via* the mother's circulation, but due to the lack of transfer through the milk, the risk would be reduced during the lactation period. Additionally, the toxicity of TBT depends on its bioavailability, for which data are lacking [121].

OTs exposure is one risk factor in reproductive function that exists for both mammalian genders. Although several investigations have shown damage to male reproductive systems [17,21,94–107,109], the adverse effects that are induced by OTs in females have been of particular concern during pregnancy [9,94,108,121]. The most critical period seems to occur during gestation because the placenta and other fetal tissues are absorbing OTs from maternal circulation and accumulating them within their tissues, inducing toxic effects such as decreases in the weights of sex organs, an imbalance in sexual hormones in the hypothalamic-pituitary-gonadal axis, alteration in the role of sexual enzymes and abnormal fetal development.

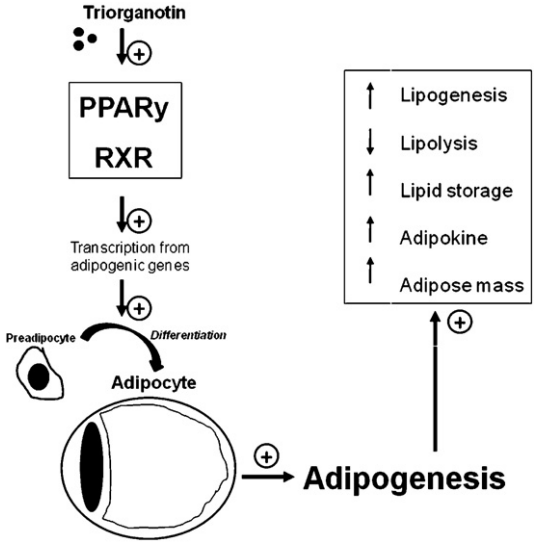

Fig. 2. The potential interaction of triorganotins with PPARγ and RXR pathways for adipogenesis. The dashed continuous arrows indicate a stimulatory (+) effect. RXR: retinoid X receptor; PPARγ: peroxisome proliferator-activated receptor gamma.

5. Organotins as obesogens and immunotoxins

Obesogens are functionally defined as chemicals (natural, pharmaceutical, or xenobiotic) that promote obesity by increasing the number of fat cells or the storage of fat in the existing fat cells [45,122,123]. They can also indirectly act on adipocytes by changing the basal metabolic rate, by shifting energy balance to favor the storage of calories, and by altering hormonal control of appetite and satiety [116,123–125]. Animal obesogens have been identified in recent years as estrogens [126], including organotins such as tributyltin [8] and bisphenol A [127]. TBTCl is an obesogen because it induces adipogenesis in the murine preadipocyte cell line at doses of 1–100 nm [8] and 10 and 100 μm [128] after 24 h of exposure. TBTCl also increases adipose mass *in vivo* in two vertebrate model organisms, those being frogs and mice [8].

Members of the PPRA subfamily of nuclear hormone receptors (PPARα, PPARβ and PPARγ) act by controlling networks of target genes along a metabolic axis [129]. PPARα is predominantly expressed in the liver and to a lesser extent in the heart and the muscles, where it has a crucial role in controlling fatty acid oxidation [130]. PPARβ (which is present throughout the body, but at low levels in the liver) is a powerful regulator of fatty catabolism and energy homeostasis [131]. PPARγ (which is present in the liver and adipose tissue) is a master regulator of the formation of fat cells and oversees their ability to function normally in adults [132]. Additionally, PPARγ is induced during adipocyte differentiation, and forced expression of PPARγ in nonadipogenic cells causes them to develop into mature adipocytes [133].

TBTCl promotes adipogenesis *in vitro* and *in vivo* by modulating key transcriptional pathways pertaining to the RXR: PPARγ heterodimer (Fig. 2), as well as C/EBPs (CCAAT/enhancer binding proteins), aP2 (adipocyte-specific fatty acid-binding protein) [8,134], Fatp (fatty acid transport protein), and more [8]. Exposing 3T3-L1 cells to TBTCl (at 10 and 100 μM for 24 h) increases adipocyte differentiation, lipid accumulation and aP2 mRNA expression (by approximately 5-fold) [8]. After 14 days of human bone marrow cell (HBMC) exposure to TBTCl, the aP2 mRNA and protein levels increase in a concentration-dependent manner [134]. In addition, the lipid droplets and PPRAγ mRNA levels increase, and the RXRα mRNA decreases in a way that was dose- and time-dependent, also in the HBMC [134]. However, Li et al. [135] suggested that the adipogenic effects of TBT could not be blocked after treatment of the 3T3-L1 cell line with a selective PPARγ antagonist. This finding suggests that TBT induces adipogenesis through different pathways at low doses, such as RXR or its other anti-estrogenic effects [128]. Mice 6-week-old (wks) dosed for 24 h with TBTCl (0.3 mg/kg *ip*) show decreased RXR, PPARγ, and C/EBPα expression in adipose tissue and testis, but increased amounts of C/EBPβ and the proadipogenic transcription factor Srebf1 in the liver and testis. In addition, Fatp mRNA levels and phosphoenol-pyruvate carboxykinase 1 expression are upregulated by 2- to 3-fold in liver and epididymal adipose tissues, but not in the testis [8]. Similarly, acetyl-coenzyme A carboxylase and fatty acid synthase are also upregulated in the liver with similar treatment, suggesting a higher fatty acid uptake and triglyceride synthesis by TBT [136–143].

In one study, male pubertal mice were treated orally with TBTCl (at 0.5, 5, and 50 μg/kg) once every 3 days for a total of 45 days. The mice showed an increase in body weight, fat mass, insulin and leptin levels, and severe hepatocyte degeneration (with ghost structures and lipid droplets which are related to the reduction of hepatic adiponectin levels), the bulk of which increased in a dose-dependent manner [144]. However, the female offspring of dams exposed to 125 ppm TBTCl (thereby feeding their offspring 125 ppm TBTCl through the placenta and their milk), show a body weight that is lower than that of controls from 9 to 15 weeks of age [145]. Pregnant monkeys showed a decrease in body weight gain at 3.8 mg/kg during 30 days of treatment after DBTCl exposure (at 0, 2.5 or 3.8 mg/kg) [116]. Although the literature demonstrates that the effects of TBTCl administration during gestation are controversial [116,144,145], TBTCl has been shown conclusively to alter the profiles of both genders of pups from GD 8 until adulthood [146].

Although there are differences in the adipogenic TBT-induced data *in vivo* and *in vitro*, the effects of TBTCl in rodent adipose tissue are dose-, gender- and age-specific [128]. Both genders of mice (at 3 wks old) that have been fed diets containing TBTCl (0, 0.05, 0.5, 5, 50, or 500 μg/kg/day) show an increase in their fat mass at a dose of 0.5 μg/kg. The increase in male mice is maintained up to the end of the exposure (day 105), and until an age of 9 wks, these mice show an increase in their fat masses until day 30. Additionally, an increased number of blood vessels with adipocytes of different sizes and shapes are observed in the epididymal tissue. In females, TBTCl produces a transient increase in fat tissue weight that is not maintained after day 60 of exposure [128]. All the investigations that showed a morphofunctional change that could be induced by organotins in mammalian metabolic function are listed in Table 1.

Based on the programming of fetal studies, maternal TBT exposure is related to pup metabolism [8,109,116,147] and immunological [134,148,149] disorders, as well as the development of obesity, among other toxic effects.

Pregnant mice that are exposed to TBTCl (at 0.05 or 0.5 mg/kg *ip*) from GD 12 to 18 have pups with increased lipid accumulation or mature adipocytes in their adipose deposits, liver, testis and mammary adipose regions, which results in raised epididymal adipose mass in mice adults [8]. Stromal cells that are isolated from white epididymal/ovarian fat pads of 8 wks mice (mADSCs) that have been exposed *in utero* to PPARγ agonists (ROSI 1 mg/kg) and TBTCl (0.1 mg/kg) exhibit an increase in their adipogenic differentiation capacities (~20%), to the detriment of osteogenic differentiation in mADSCs, with an increase in lipid accumulation (~55%); and increase in the expression of PPARγ mRNAs, Fapb4 (the early adipogenic markers were ~2-fold) and LEP (the later adipogenic markers were ~4-fold); and a decrease in adipogenesis inhibitor Pref-1 (~2.5-fold) [147]. Similarly, TBTCl stimulates the formation of adipocytes in human bone marrow stromal cells but at the same time, inhibits the formation of crucial hematopoietic factors (CKβ8-1~3.2; MIP-3α~2.4; MIP-1δ~2.0; NAP-2~3.0; IGFBPs~2.4; TGF-β~3.0), most notably of leptin (~2.0) [134].

Table 1
Morphofunctional changes induced by organotins in the metabolic function of mammals.

Model	Exposure				Findings	References
	Route/Method	Period	Duration/Time	Dose/Concentration		
Rat	TBT (in the food)	*In utero*	Pregnancy	125 ppm	↓ Mean body weight at birth ↓ Mean relative weight ↓ Activity on behavioral tests Loss on 7.6 and 15.2 mg/kg group	[145]
Rat	DBT (gastric intubation)	*In utero*	GD 0–3, GD 4–7	3.8; 7.6; or 15.2 mg/kg	Loss on 15.2 mg/kg of 4.7 days group ↓ Weight gain during pregnancy at 15.2 mg/kg ↓ Food consumption at 15.2 mg/kg	[125]
Monkey	DBT (nasogastric intubation)	GD 20–50	30 days	0; 2.5 or 3.8 mg/kg	↓ Body weight gain at 3.8 mg/kg ↓ Food consumption at 2.5 and 3.8 mg/kg	[116]
Rat	TPT (oral)	*In utero* and until weaning or puberty	GD 6 until PND 21 or PND 61	2 or 6 mg TPT/kg b.w	↓ Body weight and reproductive organ weight	[109]
Mice	TBT (oral)	Puberty	45 days	0.5; 5 and 50 μg/kg	Hepatic steatosis ↑ Body weight (5 mg/kg), hiperinsulinemia and hiperleptinemia ↑ n° of lipid droplets in the Sertoli cells	[144]
Rat	TBT (oral)	*In utero*	GD: 0–19 or 8–19	20 mg/kg	Abnormally dilated endoplasmic reticulum in Sertoli cells and gonocytes TBT 50 nM doubled the n° of cells containing	[96]
Human and mice	TBT (oral/*in vitro*)	*In utero*/Weaning and *in vitro* (Mice) *In vitro* (Human)	Pregnancy until 20–22 days of weaning Cell culture for 14 or 21 days	TBT (oral): 0.1 mg/kg TBT (cell cult.): 5, 50 nM; 50 nM of the RXR agonist (AGN)	TBT-induced the appearance of lipid droplets in the cells, as did the PPARγ agonist ROSI, TBT at 5 nM had no effect These effects where blocked by the PPARγ antagonist ↓ Dose to acutely upregulate the liver's fat storage-related enzime Adipose-derived stromal stem cells, from TBT-treated rats had a 2-fold capacity to storage fat Also there was a 2-fold expression of Fapb4	[147]
HMBC	TBT (*in vitro*)	*In vitro*	14 days	0.0007–0.07 μM	TBT also induced a decrease in some chemokines, interleukins, and growth factors ↓ of leptin expression and ↓ mean body weight ↑ n° of adipocytes	[134]
Rat	TBT (oral)	*In utero*/puberty	GD: 8 to adulthood	0.025 mg/Kg	Mean ↑ in slope of the growth curve for both gender until even lower dosages	[146]
Rat	TBT (oral)	*In utero*/weaning	GD 8–20, PND 6 or 12	10 mg/kg	↓ Liver, spleen, and thymes weights in females ↓ Dam's and pup's weights ↓ Liver weight in females Accumulation of TBT and DBT in tissues and placenta	[108]
Rat	DTOC (oral)	*In utero* until adulthood	GD 0 to PND 70	30 mg/kg	↓ n° of, CD4 and CD8 in spleen and thymus ↓ T:B cells ratio, ↑ production of IL-13 and IL-4	[148]
Mice	TBT (gavage/*In vitro*)	Adulthood/*In vitro*	30 days/24 h	0.5 μg/kg (Adulthood) 500, or 5000 μg/kg (*In vitro*)	↑ Fat mass/body weight, ↑ vessel number in epididymal WAT ↓ ER basal function at low doses (5 μg) activated estrogen responsive UCP-1 gene at high doses (50–500 μg)	[128]
SW872 cells	TMT (*in vitro*)	*In vitro*	48 h	2.5, 5, 10, 25, 50, or 100 μM	↑ TNF-α, IL-6; and ↓ leptin	[149]
Obese mice	TMT (*in vitro*)	*In vitro*	1 day	2.6 mg/kg bw	↑ TNF-α in hippocampus ↑TNF-α, MIP-1a and adiponectin mRNA levels in WAT	[149]
Cell culture	MBT, DBT, TBT, Tetra-BT (transfection/*in vitro*)	*In vitro*	24 h	0.1–10^5 nM	Activation of human RXR and PPARγ receptors by di, tri and tetrabutyltin compounds	[8]

Table 1 (*Continued*)

Model	Exposure				Findings	References
	Route/Method	Period	Duration/Time	Dose/Concentration		
3T3-L1 cells	TBT (*in vitro*)	*In vitro*	1 week	1–100 Nm	↑ n° of adipocytes 45-fold increased levels of m RNA for aP2 after 72 h exposure	
		Adulthood	24 h	0.3 mg/kg	2–3 time fold upregulation of Fatp in liver and epididymal adipose tissue ↑ of Acac, Fasn and PEPCK/Pck1	
Mice	TBT (*ip* injection)	*In utero*		0.05 or 0.5 mg/kg	After 10 weeks, the pups had no total body weight difference, but had a 20% ↑ in adipose mass	

TPT (triphenyltin chloride), DBT (dibutyltin dichloride), TMT (trimethyltin), Tetrabutyltin (tretaBT), DTOC (di-n-octyltin dichloride), HMBC (human bone marrow culture), ER (estrogen receptor), white adipose tissue (WAT), RXR (retinoid X receptor), PPARγ (peroxisome proliferator-activated receptor gamma), 3T3-L1 Cells (murine preadipocyte cell), SW872 Cells (adipocytes), UCP-1 (uncoupling protein - 1), aP2 (adipocyte-specific fatty acid-binding protein), Fatp (fatty acid transport protein), phosphoenol-pyruvate carboxykinase 1 (PEPCK/Pck1), acetyl-coenzyme A carboxylase (Acac), fatty acid synthase (Fasn), *ip* (intraperitoneal).

In addition to its adverse effects on the metabolic axis, TBTCl has also been found to reduce the spleen and thymus weights in pups from GD 8 until female PND 60 at doses of 2.5 mg/kg and male PND 30 at doses of 0.25 and 2.5 mg/kg [146]. Similar experimental models have shown thymus cortical (in terms of decreased numbers of cortical lymphocytes) and spleen atrophy in males at PND 30 at doses of 0.25 and 2.5 mg/kg. Additionally, thymus atrophy has been shown in females at PND 30 after TBTCl administration at a dose of 0.25 mg/kg, with spleen atrophy in females at PND 60 administered 0.025 and 0.25 mg/kg TBTCl [150]. These changes in both pup genders are indicative of effects on immune function [146]. The thymus is the site of T-lymphocyte maturation and differentiation, which is a process that involves the intact thymic cortex and medulla [151]. Toxic insult during immune system development may result in lifelong immunosuppression [152], and may cause increased sensitivity as a consequence of TBTCl exposure *in utero* [146]. In conjunction with these data, DOCT (0, 3, 10 or 30 mg/kg di-n-octyltin dichloride or DOTC) exposure in immature (PND 10–21) and near-adult (PND 10–42) immune systems causes alterations in immune tissue morphology (a decreased number of CD 4s and CD 8s in the spleen and thymus) and immune function [148,153].

In reviewing the involvement of TBT during *in utero* immune development, previous studies have also shown that a mammal's immune system could be the target organ of TBT toxicity [134,148,149]. Penninks [154] reported that short-term exposure to TBTCl reduced the body weight of the exposed animals and caused morphologic changes to the lymphoid tissue (particularly the thymus). In addition, depletion of lymphocytes in the spleen and lymph nodes, lymphopenia and alteration of serum immunoglobulin levels has been observed under these conditions [154,155]. It has been proposed that thymus atrophy may be related to the inhibition of immature thymocyte proliferation [156]. TBTCl has been reported to induce apoptosis, possibly as a result of a rapid increase in free cytosolic Ca^{2+} levels [157]. Thymocite apoptosis plays a crucial role in T cell selection and immune regulation [158]. Thymocyte apoptosis may be mediated by Fas expression in 4 wks mice after TBTCl exposure for 28 days at doses of 4 and 20 mg/kg, suppressing both the humoral and cellular immune responses [159].

Mice treated orally with TPTCl (69 mg/kg) and TBTCl (63 mg/kg) experience a reduction in peripheral lymphocytes at 3–6 h, and thymus atrophy is observed at 6 and 24 h after exposure. In isolated lymphocites incubated with 500 ng/ml of TPTCl and TBTCl, necrosis in over 70% of the treated cells has been observed [160]. In addition to modulating cytokine release, such as TNF-α and INF-δ, TBTCl induces a dose-related (0.001, 0.01, 0.1, 1 and 10 μM) decrease in the colony number of human cord blood cells *in vitro* after 14 days of exposure. Additionally, after 24 h of TBTCl treatment, rodent spleen cell mitogen that was stimulated with lipopolysaccharide from *Escherichia coli* (1 μg/ml) is reduced in addition to phytohemagglutinin (PHA, 1.2 μg/ml) after 48 h [161]. In contrast, exposing both genders of rats to dimethyltin dichloride (DMTCl) (at 0, 1.7 or 3.4 mg/kg for 28 days), along with pregnant females, pups at GD 6 (0, 2.4 or 4.6 mg/kg until weaning) and PND 2 (0, 3.6 or 6.9 mg/kg) does not cause immune function effects [162].

Although triorganotins are able to modulate adipogenesis and/or the immune system, it is now well established that the physiological activity of white adipose tissue (WAT) is greater than initially thought, with cross talk and further involvement with other systems, such as the immune system [163], after xenobiotic exposure. Ravanan et al. [149] demonstrated that human adipocytes and macrophages were resistant to cell death as induced by 100 μM trimethyltin hydroxide (TMT) for 48 h. However, the adipocytes showed increased expression of TNF-α and IL-6 mRNA and modified leptin levels. In macrophage cultures, TMT increased TNF-α and IL-6 levels, while MCP-1 and MIP-1α levels were decreased. In mice, a single injection of TBT elevated the TNF-α, MIP-1α and adiponectin in WAT after 24 h. Macrophages are a primary source of inflammatory cytokines in tissues, and in WAT, the number of infiltrating macrophages is related to the adipose tissue mass [164]. It has been proposed that the balance between M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages within a tissue influences the inflammatory response [165]. These data suggest that WAT can serve as a pool for inflammatory cytokine production, acutely responding to exposure to an organotin, such as TMT [149].

Another possible cause of changes to the metabolic and immune function related to the development of obesity in both humans and mice could be shifts in gut microbiota [166,167,168]. In addition, it has been shown that the transfer of gut microbiota from obese (*ob/ob*) mice to germ-free wild-type (WT) recipients leads to an increase in fat mass in wild-type mice [169]. The innate immune system participates in regulating the gut microbiota [170]. During infection/inflammation, the innate immune system may play a key role in promoting metabolic heath. Vijay-Kumar et al. [168] showed that a transfer of the gut microbiota from TLR5-deficient mice to the WT leads to hyperphagia, obesity (increase fat pad), hyperglycemia, insulin resistance, colomegaly and elevated levels of pro-inflammatory cytokines (TNF-α and IL-1β). The toxic effects of OTs on bacteria include the inhibition of amino acid uptake and of cell growth [171,172]. In *E. coli* (a resident of the human small intestine), membrane-bound ATPase and energy-dependent pyridine dinucleotide transhydrogenase are inhibited by TBTCl [173]. It is possible that organotins may alter the mammalian gut bacteria profile and could induce the development of metabolic disorders, but this claim needs to be further investigated.

In short, TBT stands as a predominant factor in the obesity puzzle because evidence supports its roles in increased adipocyte

differentiation [23,147], activation of fat storage-related nuclear receptors and proteins [147], disrupted levels of chemokines, interleukins [134], leptin [134,149], and insulin [144], and lower body weight at birth [145]. Exposure to this compound represents a potential health risk.

6. Human exposure

For the general population, the major route of exposure to most organotins is ingestion, through the consumption of either contaminated drinking water or beverages, or by eating particular marine foods [61,174]. Marine fishery products may contain high TBT levels [175], and different diets are expected to result in different organotin loads in human tissues [120,176,177]. However, in spite of the evidence that such sources expose humans to OTs, limited data on butyltin (BT) deposition in humans are available. Thus, human risk assessment has mainly been based on immunological studies in experimental animals and estimated human intake of seafood sources [175]. For example, the intake of BT from seafood consumption by the South Korean population was estimated to be 17.2 ng/kg body weight/day. Fish accounted for 50% of their total BT intake, and the next contributors were cephalopods (26%). The estimated intakes for males and females were 18.6 and 15.7 ng/kg body weight/day, respectively. Among the age groups investigated, children (2 and 3–6 years) had higher intakes of BTs than adults. The estimated intake of BT for the Korean population was 5–8% of the tolerable daily intake of 250 ng/kg body weight/day as set by the European Food Safety Authority [178].

Based on immune function studies, a Tolerable Daily Intake (TDI) value for TBT of 0.25 μg $(kg\,bw)^{-1}$ day^{-1} was established [154]. Because of uncertainties in human–rat toxicity extrapolation, human–rat kinetics extrapolation, and inter-individual differences in both toxicity and kinetics, a safety factor of 100 was used for the final calculation of the TDI. This TDI was based solely on the reduced thymus weights that resulted from feeding TBT to adult rats. Furthermore, this TDI has been adopted by the WHO [179]. In humans, daily TBT intake was estimated to range from 2.2 to 6.9 μg/individual [180,181]. However, the information on human exposure to BT compounds is also limited. In general, DBT appears to be the main BT species that is deposited in the human liver [182]. The danger posed by OTs to humans depends not only on their solubilization but also on the possibility that they may degrade during human digestion. It has been found that the permeability pattern of BT in the Caco-2 *in vitro* intestinal cell line correlates well with the general *in vivo* toxicity pattern (trialkyltin > dialkyltin > monoalkyltin), but is different from the accumulation pattern (DBT > TBT > MBT) [183]. It has also been suggested that the high accumulation of DBT in Caco-2 cells may result from its strong chemical affinity for the dithiol that is found in many enzymes [184] and that is not found during the degradation of TBT to DBT and MBT [183]. As in human enterocytes, Caco-2 cells fail to express the predominant enzymes of the cytochrome P450 family, when exposed to TBT and DBT [185].

Kirchner et al. [147] demonstrated this effect in human blood and tissue samples in the range of 3–100 mM of TBT exposure. In contrast, an average TBTCl concentration of 27 nM is sufficient to activate RXR and PPARy in mice [8]. These tissue and serum levels appear to be higher in societies that consume large amounts of fish; TBT levels in the livers of Japanese people were in the range of 56–96 ng/g wet wt [8,147]. Studies have shown that aromatase mRNA levels can be downregulated in human ovarian granulosa cells by OTs or the ligands for RXRs or PPARy [186–188]. The binding of TBTCl to these nuclear receptors may indicate relationship between reproductive and metabolic disorders [128].

Seafood samples collected from markets in Asian, European and North American cities have been shown to have TBT levels averaging 185 ng $(g\,dw)^{-1}$. Daily intakes of TBT have been determined in Japan by the duplicated portion method, and the values were found to be $4.7 \pm 7.0\,\mu g/d^{-1}$ in 1991 and $2.2 \pm 2.2\,\mu g/d^{-1}$ in 1992. Using the market based method, the daily intake was estimated at 6–9 $\mu g/d^{-1}$ in 1991 and 6–7 $\mu g/d^{-1}$ in 1992 [180]. Based on the average per capita seafood consumption rates for each country, the amounts of TBT ingested has not been found to exceed proposed thresholds for chronic effects, suggesting negligible risks for the average consumer [15,90]. In Taiwan, TBT levels in various oysters ranging from 320 to 1510 ng/g dry wt. have been found, varying by sampling location. The highest TBT levels (where TBT presented the major composition of total BT compounds or 86–91%) were found in oysters at 1510 ng/g dry wt. from oysters in the Hsiangshan coastal area. The values of exposure *via* oyster consumption for fishermen have been shown to be 94.1 and 250 g/d for typically and maximally exposed individuals, respectively. In particular, the highest intake (250 g/d) from fishermen has been shown to be almost two times greater than that of the general population (139 g/d). These results indicate that people who are exposed to contaminated oysters or other seafood could face potential health risks [61,177] as well as persons weighing less, *e.g.*, children [189].

TBT is generally toxic to mammalian cells because it causes apoptosis or necrosis [47,190]. It seems that OTs are potential modulators of human placental estrogen biosynthesis and human chorionic gonadotropin production *in vitro*, and the placenta represents a potential target organ in pregnant women [190] and rodents [108]. With respect to mechanisms of action, several biochemical processes have been identified as targets for TBT and some of these are involved in fundamental processes such as mitochondrial respiration, ion channels, steroidogenesis, receptor activation, and gene transcription [121,191]. Investigations of experimental toxicity, dietary intake, potential human health effects and the development of new sustainable technologies to remove OTs that are coupled with new sampling approaches are clearly necessary.

7. Conclusion

Exposure to triorganotins, especially TBT, induced impairments in immunological, reproductive and metabolic function *in vivo* and *in vitro*. OTs are potent, endocrine-disrupting chemicals that act against marine invertebrates, mainly (but not exclusively) mollusks. In addition, OTs are also suspected to cause imbalances in the endocrine systems of mammals, including humans and rodents, at least in part as a consequence of this compound's bioaccumulation along the food chain, starting with the intake of contaminated seafood. The toxicity level of OT may be related to its concentration, the timing of exposure, bioavailability, and sensitivity of the biota, as well as to the persistence of various compounds in the environment. TBT cause disorders in the mammalian reproductive system, such the impairment of the modulation of the hypothalamic-pituitary-gonadal axis (ovary and testis). Moreover, TBTs can cross the maternal-fetal-placental unit, inducing physiological and morphological changes, which lead to abnormal fetal and postnatal development. In addition, recent investigations suggest that organotins are obesogenic chemicals and that TBT is an example of an environmental endocrine disruptor that promotes adipogenesis through RXR and PPARy activation *in vitro* and *in vitro*. This compound is correlated with changes in the immune system because it modulates the release of cytokines. OTs can alter homeostatic metabolic set-points, disrupt appetite controls, perturb lipid homeostasis to promote adipocyte hypertrophy, and stimulate adipogenic pathways that enhance adipocyte hyperplasia. Improved studies on absorption, distribution, metabolism and elimination are required in different animal models. Thus, environmental monitoring is necessary to prevent severe effects and because it is important

to decrease the application of organotins in industrial sectors, particularly in antifouling paints, it is necessary to reduce the risk of disease development and to protect marine animal, human and environmental health.

Conflict of interest

None.

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