RAFAELA PIRES ERTHAL

BAIXAS DOSES DE MALATION PREJUDICAM O DESENVOLVIMENTO PÓS-NATAL DOS SISTEMA GENITAL MASCULINO E FEMININO DE RATOS:

MODELOS *in vitro* E *in vivo*

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BANCA EXAMINADORA

_____

Orientadora: Profa Dra. Glaura Scantamburlo Alves Fernandes
Universidade Estadual de Londrina - UEL

_____

Profa Dra. Alessandra Lourenço Cecchini Armani
Universidade Estadual de Londrina - UEL

_____

Profa Dra. Juliana Elaine Perobelli
Universidade Federal de São Paulo – UNIFESP

_____

Profa Dra. Glaucia Eloisa Munhoz de Lion Siervo
Universidade Federal do Triângulo Mineiro - UFTM

_____

Profa Dra. Flávia Karina Delella
Universidade Estadual Paulista - UNESP

Londrina, 07 de julho de 2022.

Dedico este trabalho,

Aos meus pais, que desde meus primeiros passos me incentivaram a enxergar além e a lutar por meus objetivos.

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“Um pouco de ciência nos afasta de Deus.
Muita, nos aproxima.”

Louis Pasteur

ERTHAL, Rafaela Pires. **Baixas doses de malation prejudica o desenvolvimento pós-natal dos sistema genital feminino e masculino de ratos:** Modelos *in vitro* e *in vivo*. 2022. 168 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2022.

RESUMO

O malation é um inseticida da classe dos organofosforados amplamente utilizado na cultura agrícola e no meio urbano para controle das arboviroses, causados pelo mosquito transmissor *Aedes aegypt*. Crianças e adolescentes estão sendo expostos a esses compostos através da ingestão de alimentos contaminados ou inalação de gotículas. Diante da falta de informações específicas na literatura e da relevância clínica, social e política do assunto, o objetivo do presente trabalho foi avaliar se a exposição a baixas doses de malation pode comprometer a qualidade de espermatozoides, de folículos ovarianos, a função de células de Leydig e o desenvolvimento do sistema genital feminino. Para avaliar a qualidade espermática, 24 ratos machos foram organizados em 3 grupos experimentais: controle (veículo) e malation 10 (M10) ou 50 mg/kg (M50), doses consideradas baixas para a exposição animal. Os animais foram expostos do dia pós natal (DPN) 25-65. Ao final, foram submetidos à eutanásia para coleta de testículos, destinados à avaliação da expressão gênica, e de espermatozoides a partir dos epidídimos para posterior avaliação da função espermática e perfil oxidativo. Foi observada redução na expressão dos genes 17-ßHSD e de Receptores de Andrógenos (AR) em testículos, bem como comprometimento da integridade acrossômica de espermatozoides associado a perturbação na atividade de enzimas antioxidantes. Para avaliar o impacto do malation sobre a função de células de Leydig, células de Leydig da linhagem murina TM3 foram expostas às concentrações de 1, 10 ou 100 µM de malation por 24 horas. Após a exposição, foram avaliadas a viabilidade celular, concentrações de testosterona e de citocinas a partir do meio de cultura, bem como avaliação do perfil redox das células. Nessas condições experimentais, o malation comprometeu a viabilidade celular, reduziu a produção de testosterona pelas células de Leydig nas menores concentrações experimentais, desequilibrou os níveis de citocinas e o perfil oxidativo dessas células. Concluímos portanto que, o malation compromete a função das células de Leydig através da alteração do estado redox celular. Para avaliar os efeitos específicos do malation sobre o desenvolvimento de folículos pré-antrais, foi utilizado modelo *in vitro* em que fragmentos ovarianos provenientes de vacas Nelore foram cultivados por 44 horas em MEM suplementado com malation (500 ou 1000 µM). Após o cultivo, os fragmentos foram destinados à avaliação histológica e de perfil oxidativo, enquanto que o MEM foi utilizado para dosagem de estradiol. Nesse modelo, embora o perfil oxidativo não tenha sido alterado pela exposição ao malation, os níveis de estradiol foram reduzidos e foi observado comprometimento na integridade e morfometria de folículos pré-antrais. O último experimento abordou os efeitos de baixas doses de malation sobre o desenvolvimento pós-natal do sistema genital feminino. Para tanto, 30 ratas foram organizadas nos grupos controle, M10 ou M50 e expostas entre o DPN 22 e 60. Durante esse período, foi avaliada a instalação da puberdade. Ao final do período experimental, os animais foram submetidos à eutanásia para coleta de sangue destinado à dosagem de estradiol e de ovários e útero destinados à avaliação

histológica, de perfil oxidativo e expressão gênica. Nesse modelo, embora a instalação da puberdade e os níveis de estradiol não tenham sido prejudicados pela exposição a baixas doses de malation, o inseticida comprometeu a integridade e morfometria ovariana e uterina através da alteração do perfil oxidativo e da expressão de genes relacionados com a regulação do ciclo celular, antiapoptóticos e de ação endócrina. A partir dos estudos supracitados, concluímos que o malation pode prejudicar a integridade de folículos e o desenvolvimento pós-natal dos sistemas reprodutor feminino e masculino através da alteração de perfil oxidativo e expressão de genes envolvidos nas vias de sinalização endócrina e de controle de proliferação celular.

Palavras-chave: organofosforado; puberdade; ovário; útero; espermatozoide; estresse oxidativo; expressão gênica.

ERTHAL, Rafaela Pires. **Low doses of malathion impair the postnatal development of the male and female genital systems of rats:** *in vitro* And *in vivo* Models. 2022. 168 p. Thesis (Doctorate in Experimental Pathology) – State University of Londrina, Londrina, 2021.

ABSTRACT

Malation is an organophosphate insecticide widely used in agricultural and urban settings to control arboviroses caused by the mosquito vector Aedes aegypt. Children and adolescents are being exposed to these compounds through ingestion of contaminated food or inhalation of containing droplets. Given the lack of specific information in the literature and the clinical, social and political relevance of the subject, the objective of the present study was to evaluate whether exposure to low doses of malation can compromise sperm quality, ovarian follicles, Leydig cell function and the development of the female genital system. To evaluate sperm quality, 24 male rats were organized into 3 experimental groups: control (vehicle) and malathion 10 (M10) or 50 mg/kg (M50), doses considered low for animal exposure. The animals were exposed from post natal day (PND) 25-65. At the end, they were euthanized to collect testes for gene expression evaluation and spermatozoa from the epididymis for further evaluation of sperm function and oxidative profile. Reduced expression of 17-ßHSD and Androgen Receptor (AR) genes was observed in testes, as well as impaired acrosomal integrity of spermatozoa associated with disturbed antioxidant enzyme activity. To evaluate the impact of malathion on Leydig cell function, Leydig cells of the murine TM3 strain were exposed to concentrations of 1, 10 or 100 µM malathion for 24 hours. After exposure, cell viability, testosterone and cytokine concentrations from the culture medium, as well as evaluation of the redox profile of the cells were evaluated. Under these experimental conditions, malathion compromised cell viability, reduced testosterone production by Leydig cells at the lowest experimental concentrations, unbalanced cytokine levels and the oxidative profile of these cells. We therefore conclude that, malathion compromises Leydig cell function by altering the cellular redox state. To evaluate the specific effects of malathion on preantral follicle development, we used an in vitro model in which ovarian fragments from Nelore cows were cultured for 44 hours in MEM supplemented with malathion (500 or 1000 µM). After cultivation, the fragments were used for histological and oxidative profile evaluation, while MEM was used for estradiol dosage. In this model, although the oxidative profile was not altered by malation exposure, estradiol levels were reduced and impairment in preantral follicle integrity and morphometry was observed. The last experiment addressed the effects of low doses of malation on the postnatal development of the female genital system. For this purpose, 30 rats were organized in control, M10 or M50 groups and exposed between PND 22 and 60. During this period, the onset of puberty was evaluated. At the end of the experimental period, the animals were euthanized to collect blood for estradiol dosage and ovaries and uterus for histological evaluation, oxidative profile and gene expression. In this model, although the onset of puberty and estradiol levels were not affected by exposure to low doses of malathion, the insecticide compromised the ovarian and uterine integrity and morphometry by altering the oxidative profile and the expression of genes related to cell cycle regulation, antiapoptotic and endocrine action. From the aforementioned studies, we conclude that malathion may impair follicle integrity and postnatal development of the female and male reproductive systems through alteration of

oxidative profile and expression of genes involved in endocrine signaling and cell proliferation control pathways. Translated with www.DeepL.com/Translator (free version)

Key words: organophosphate; puberty; ovary; uterus; sperm; oxidative stress; gene expression.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

-1O2	Oxigênio Singlet
ABRASCO	Associação Brasileira de Saúde Coletiva
AChE	Acetilcolinesterase
ANVISA	Agência Nacional de Vigilância Sanitária
AR	Receptor de Andrógenos
AREs	Elementos Responsivos a Andrógenos
ATSDR	Agency for Toxic Substances and Disease Registry (Agência para Substâncias Tóxicas e Registro de Doenças)
BHT	Barreira Hematotesticular
CONAMA	Conselho Nacional do Meio Ambiente
DPN	Dia Pós-Natal
EROs	Espécies Reativas de Oxigênio
FAO	Food and Agricultura Organization (Organização da Comida e Agricultura)
FSH	Follicle Stimulating Hormone (Hormônio Folículo Estimulante)
GnRH	Gonadotropin-Releasing Hormone (Hormônio Liberador de Gonadotrofinas)
GSH	Glutationa reduzida
H2O2	Peróxido de Hidrogênio
HHG	Eixo hipotalâmico-hipofisário-gonadal
IARC	Agência Internacional para Pesquisa do Câncer
INCA	Instituto Nacional de Câncer José Alencar Gomes da Silva
IOC	Instituto Oswaldo Cruz
LH	Luteinizing Hormone (Hormônio Luteinizante)
LHR	Luteinizing Hormone Receptor (Receptor de hormônio luteinizante)
LOOH	Hidroperóxidos lipídicos
MDA	Malondialdeído
O2-	Ânion Superóxido
OFs	Organofosforados
-OH	Radical Hidroxila
OMS	Organização Mundial da Saúde
PLOOH	Hidroperóxidos fosfolipídicos

SOD	Superóxido Dismutase
StAR	Steroidogenic acute regulatory protein (Proteína Reguladora Aguda Esteroidogênica)

SUMÁRIO

1 REVISÃO DE LITERATURA

1.1 AGROTÓXICOS E ORGANOFOSFORADOS

No Brasil, o uso de agrotóxicos é normatizado pela Agência Nacional de Vigilância Sanitária (ANVISA) e pelo Conselho Nacional do Meio Ambiente (CONAMA). Desde 2008, o país ocupa posição de destaque como maior consumidos mundial de agrotóxicos. Com o aumento acelerado no uso de agrotóxicos, os possíveis efeitos a partir desse uso sobre o meio ambiente e a saúde humana passaram a ser discutidos. De acordo com a FAO – Food and Agriculture Organization, agrotóxicos são “produtos químicos ou quaisquer substâncias ou mistura de substâncias destinadas a prevenção, à destruição ou ao controle de qualquer praga, incluindo os vetores de doenças humanas ou de animais, que causam prejuízo ou interferem de qualquer outra forma na produção, na elaboração, na armazenagem, no transporte ou na comercialização de alimentos, para os homens ou os animais, de produtos agrícolas de madeira, ou que podem ser administrados aos animais para combater insetos, aracnídeos ou outras pragas dentro ou sobre seus corpos” (ALONZO; CORRÊA, 2003).

Os agrotóxicos podem ser classificados de acordo com seus alvos de ação em inseticidas (controle de insetos), fungicidas (controle de fungos), herbicidas (controle de plantas invasoras), fumegantes (controle de bactérias presentes no solo), rodenticidas (controle de roedores), dentre outros (RIBAS; MATSUMURA, 2009). Além disso, podem ser classificados também de acordo com a periculosidade ambiental, sendo produtos altamente perigosos ao meio ambiente (Classe I), produtos muito perigosos ao meio ambiente (Classe II), produtos perigosos ao meio ambiente (Classe III) e produtos pouco perigosos ao meio ambiente (Classe IV).

No Brasil, o uso de agrotóxicos é normatizado pela Agência Nacional de Vigilância Sanitária (ANVISA) e pelo Conselho Nacional do Meio Ambiente (CONAMA). A classificação utilizada para avaliar a toxicidade para a saúde humana, em território nacional, é a DL 50 (quantidade de uma determinada substância que é necessária ingerir ou administrar para provocar a morte a pelo menos 50% da população em estudo).

Além do aumento de danos ambientais e na saúde das pessoas em decorrências da exposição aos agrotóxicos através do consumo de alimentos contaminados, os agricultores e seus familiares são o grupo com maior potencial de exposição (PEDLOWSKI et al., 2012). Dessa maneira, a utilização dos agrotóxicos pode ter impacto direto por três vias: (1) via ocupacional, caracterizada pela contaminação dos trabalhadores que manuseiam tais compostos, (2) via ambiental, através da dispersão dessas substâncias pelo meio ambiente (lagos, contaminação atmosférica e solo) e (3) via alimentar, caracterizada pela contaminação de alimentos que serão ingeridos (MOREIRA et al., 2002).

Desde 2008, o país tem ocupado a posição de maior consumidor mundial de agrotóxicos, sendo que mais de 80% dos agricultores brasileiros fazem uso de tais produtos para combater pragas e aumentar a produtividade. De acordo com o Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX), foram registrados 4.656 casos de intoxicação por agrotóxicos no Brasil, sendo que 128 foram a óbito (SINITOX, 2012). As intoxicações agudas por agrotóxicos são as mais relatadas, afetando principalmente trabalhadores pela via ocupacional.

Ribeiro *et al.* (2007) relataram que foram encontrados resíduos de agrotóxicos em águas subterrâneos em países da Europa, Estados Unidos e Brasil. Como consequência dessa contaminação ambiental, pode-se encontrar resíduos de agrotóxicos em alimentos que serão consumidos, sendo que, muitos deles possuem atividade hormonal atuando sobre receptores de estrógenos e andrógenos (FONTENELE et al., 2010). O Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA, 2017) tem se manifestado contra práticas atuais no uso exacerbado de agrotóxicos, ressaltando seus riscos à saúde, especialmente na importância clínica para o desenvolvimento do câncer.

Dentre os agrotóxicos utilizados em larga escala, destaca-se o grupo dos organofosforados. Trata-se de compostos químicos com atividade inseticida utilizados no controle e combate a pragas. São altamente lipossolúveis e rapidamente hidrolisados (ALONZO; CORRÊA, 2003). A Figura 1 apresenta a fórmula estrutural básica dos organofosforados (OFs).

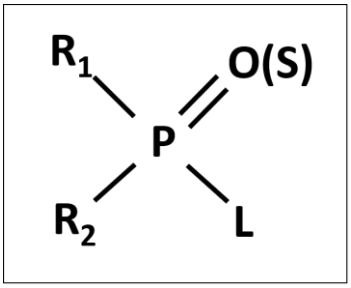

Figura 1. Fórmula estrutural básica dos compostos organofosforados. P – fósforo; O – oxigênio; S – enxofre; R_1 e R_2 representam grupos alcoxil; L – halogênios; alquil, aril e heterocíclicos (modificado de dos Santos et al., 2007).

Estes compostos podem ser absorvidos via dérmica, respiratória ou digestiva. Crianças e adultos podem estar expostos por via oral, através do manuseio de instrumentos de pulverização ou através da ingestão de alimentos contaminados (LARINI, 1996). Após serem absorvidos, os OFs agem fosforilando e inibindo de maneira irreversível as enzimas acetilcolinesterase (AChE) nas sinapses do sistema nervoso central, conforme apresentado na Figura 2. Com o acúmulo de acetilcolina nas sinapses, tem-se a interrupção da propagação de impulsos elétricos,e a consequente paralisia e morte dos insetos (BRAGA; VALLE, 2007; TAYLOR, 1996). Ressalta-se ainda que, o mesmo mecanismo acontece em aves ou mamíferos.

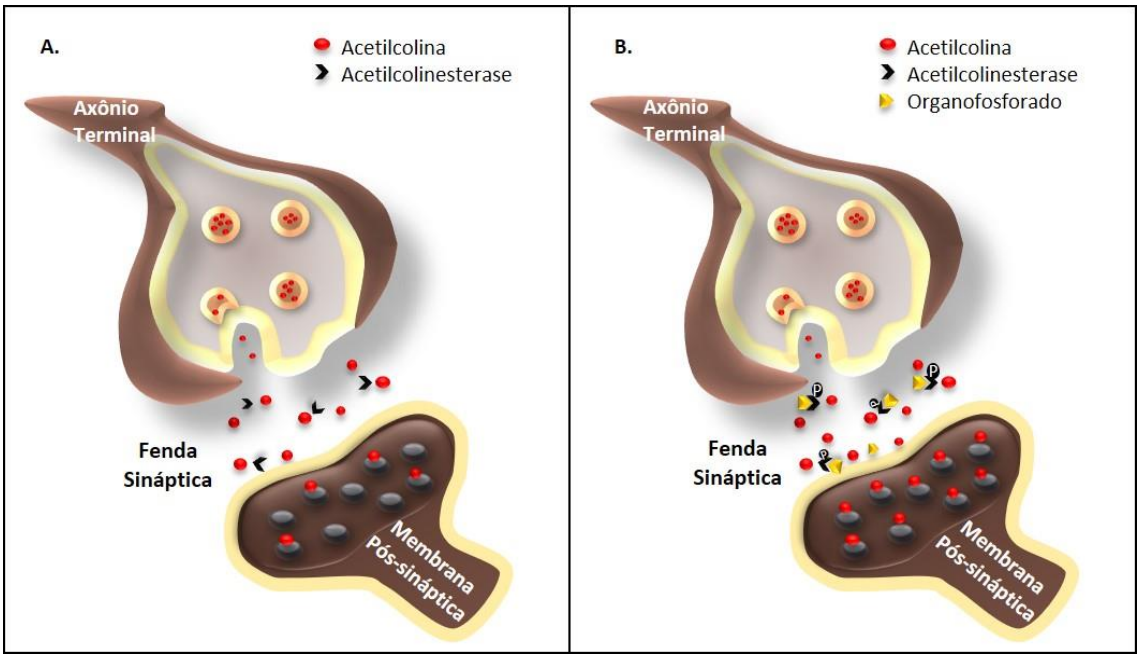

Figura 2. Mecanismo de ação dos OFs. **A.** Ação fisiológica da acetilcolina em receptor pós-sinápticos e intervenção da enzima acetilcolinesterase **B.** Organofosforado fosforilando e inibindo as enzimas acetilcolinesterase, levando ao acúmulo do neurotransmissor na fenda sináptica (ERTHAL, 2018). P – fósforo.

A sintomatologia de intoxicações agudas por OFs é explicada pelo aumento de efeitos da acetilcolina em receptor muscarínicos, caracterizado por aumento de secreções, broncoconstrição, bradicardia, vômitos e aumento da motilidade intestinal. Além disso, o aumento da ação da acetilcolina em receptores nicotínicos caracteriza o bloqueio da despolarização de membrana e consequente paralisia muscular (KARALLIEDDE; SENANAYAKE, 1989). De acordo com Kemple (2001) a exposição crônica aos organofosforados refere-se a tempo superior a 10% do tempo de vida médio de determinada espécie. Deve-se considerar esse tipo de exposição pois, embora os sinais e sintomas sejam mais brandos e discretos quando os indivíduos são expostos a baixas doses do organofosforado, a evolução de danos e sinais de toxicidade podem ser despercebidos.

1.2 MALATION

As arboviroses representadas pelas doenças causadas pelos vírus da dengue, zica e chikunkunya são grandes problemas de saúde pública no Brasil (BRASIL, 2015a). O mosquito do gênero *Aedes aegypt* é o vetor dessas doenças, sendo a fêmea de maior importância no processo da transmissão. Buscando intensificar a mobilização e combate a esse vetor, foi elaborado um Plano Nacional de Enfretamento à Microcefalia frente aos diversos casos de neonatos com microcefalia em associação com casos de infecção pelo zica vírus (BRASIL, 2015b). A medida adotada desde então, baseia-se no combate ao mosquito adulto através do uso de inseticidas químicos.

De acordo com recomendações da Organização Mundial da Saúde (OMS), cinco inseticidas foram aprovados para uso em Saúde Pública visando controlar os mosquitos vetores adultos, sendo quatro pertencentes à classe dos piretroides e o malation, pertencente ao grupo dos organofosforados (WHO, 2012). Uma nota técnica apresentada pelo Instituto Oswaldo Cruz (IOC, Fiocruz) apresentou a resistência desses mosquitos à classe dos piretroides e forneceu embasamento para que o Ministério da Saúde substituísse tal classe de compostos pelo malation, como forma de controle de mosquitos adultos a partir de 2009 (BRASIL, 2016a).

Os responsáveis ativos por fazer a manipulação do malation no Brasil são os Agentes Comunitários de Saúde (ACS) e Agentes de Combate a Endemias (ACE) (ZARA et al., 2016), sendo os mais expostos ao composto através da aplicação

de malation por meio de equipamentos portáteis costal ou vinculados a veículos pulverizadores (PESSOA et al., 2016). Mesmo que seja mandatória a utilização de EPIs por parte desses profissionais, Leme et al. (2014) constataram que as vestimentas não eram suficientes para reter 100% do composto, o qual atingia a superfície da pele desses indivíduos. Além disso, a lavagem da vestimenta não garantia total retenção do composto, representando outra fonte de contaminação ambiental. Dessa maneira, tanto os ACS quanto a população que vive em áreas onde o malation é pulverizado visando o controle de mosquitos estão altamente expostas ao inseticida malation (ATSDR, 2003).

O malation tem sido utilizado tanto no ambiente urbano quanto no rural. A Anvisa indica o uso do inseticida para aplicação folicular em culturas de alimentos, como alface, brócolis, citrus, couve, arroz, feijão, milho e trigo (ANVISA, 2012). O composto ainda tem sido utilizado para erradicação de insetos, formigas ou piolhos através de uma formulação comercial. O nome químico do composto é O,O-dimetil-ditiofosfato de dietil mercaptosuccinato e, quando puro, é encontrado no estado líquido amarelado. É pouco solúvel em água e solúvel na maioria dos compostos orgânicos. Sua meia-vida em pH 7,4 e temperatura 37,5 ºC é de aproximadamente 32 horas (EPA, 2006). Quando introduzido ao meio ambiente através da pulverização, as gotas do composto aderem ao solo, plantas, água e superfícies artificiais (ATSDR, 2003).

Possavatz et al. (2014) identificaram resíduos de malation na Bacia Hidrográfica do Rio Cuiabá, a qual é de grande importância para agasalhar o Pantanal, a maior área úmida mundial. Nesse caso, tal contaminação reflete o alto índice de atividade agrícola uma vez que a base econômica da região se baseia no cultivo de soja, algodão e milho, o qual requer uso de grande quantidade de pesticidas (MATO GROSSO, 2003; IBGE, 2013). Foram identificadas concentrações desses pesticidas, envolvendo malation, de 5,7 a 73,9 µg/kg de sedimento de rio. Foi apontado ainda que o principal meio de transporte de contaminação por malation foi dissolvido em água, sendo de suma importância para contaminação de alimentos que serão consumidos posteriormente (POSSAVATZ et al., 2014).

Jensen e Whatling (2010) afirmam que a exposição ao malation por via oral é possível através do consumo de alimentos e água contaminada. O risco potencial do consumo de malation por essa via tem sido estudada há tempos por autoridades

regulatórias como a U.S. EPA (2006), European Food Safety Authority (EFSA, 2006) e pela Joint FAO/WHO Meeting on Pesticide Residues (1999).

O malation destaca-se por sua alta eficácia como inseticida e baixa toxicidade em mamíferos quando comparado a outros compostos da classe dos OFs. A dose letal 50 (DL_{50}) do malation administrado via oral a ratos varia entre 1500 e 2000 mg/kg (EPA, 2000). De acordo com a Organização Mundial da Saúde (OMS), o malation enquadra-se à classe III (moderadamente tóxico) da classificação de pesticidas de acordo com a periculosidade (ATSDR, 2003).

Quando absorvido, o composto é metabolizado pelas enzimas hepáticas do citocromo P-450 originando o metabólito malaoxon, o principal responsável pela inibição das ChE e efeitos tóxicos citados anteriormente (EPA, 2000). Tanto o malation quando o malaoxon são rapidamente excretados nas primeiras 24 horas majoritariamente pela urina (aproximadamente 84%) e parcialmente pelas fezes (cerca de 6%) (EPA, 2000). A meia vida do malation em sangue de ratos é de 1,4 dias quando expostos por via oral (ATSDR, 2003).

Os biomarcadores mais específicos para identificação de exposição ao malation são compostos semelhantes e metabólitos em tecidos e fluidos corporais. No entanto, os testes devem ser realizados rapidamente pois seus metabólitos são excretados rapidamente (AKGUR et al., 1999; MORGADE; BARQUET, 1982). Estudos populacionais e de exposições ocupacionais detectaram ácido dicarboxílico malaoxon (DCA), ácido monocarboxílico malation (MCA), ácido fosforotióico dimetil (DMPT), O,O-dimetil-fosforoditioato (DMPDT) e O,O-dimetilfosfato (DMP) como os principais produtos metabólicos em amostras de sangue e urina (ATSDR, 2003).

Em pesquisa de aproximadamente 7000 pessoas residentes nos EUA conduzido entre 1976 e 1980, 1,1% apresentou concentrações quantificáveis de MCA em urina e <1% apresentou concentrações quantificáveis de DCA (KUTZ et al., 1992), sendo a concentração máxima relatada de 250 µg/L. Estudo de 5 trabalhadores e 16 residentes expostos ao malation em processos de pulverização no Haiti determinou níveis em urina de MCA em diversos tempos após a exposição, sendo que os residentes não estavam presentes durante a pulverização. Foi observado que os níveis urinários de MCA variaram entre 0,9 e 6,8 mg/L após uma semana de pulverização (WARREN et al., 1985).

A Food and Drug Organization (FAO) estabelece que exposição em modelos experimentais nas concentrações de 29 mg/kg de malation por dia durante dois anos

não causou toxicidade ou carcinogenicidade; 400 mg/kg por dia não levou a toxicidade materna em estudo de toxicidade durante o desenvolvimento; 130 mg/kg por dia não causou danos em estudo de toxicidade reprodutiva; 0,3 mg/kg durante 47 dias não alterou parâmetros toxicológicos em humanos. Dessa maneira, a concentração de ingestão diária considerada aceitável para humanos pelo órgão é de 0-0,3 mg/kg de peso corpóreo de malation (FAO, 1997).

A indução do estresse oxidativo pela geração de radicais livres e/ou alteração no sistema enzimático de antioxidantes tem sido apontada como um dos principais meios de toxicidade do malation (BROCARDO et al., 2005; FORTUNATO et al., 2006; KOVACIC, 2003). Silva et al. (2007) aponta ainda que diferentes vias e doses de exposição ao malation, bem como a idade dos animais e distribuição tecidual são todos fatores que podem interferir nos níveis de estresse oxidativo. Além do estresse oxidativo e da inibição da enzima AchE, os mecanismos tóxicos do malation também envolvem a indução da resposta inflamatória, apoptose, genotoxicidade e imunomodulação (BADR, 2020).

A Agência International para Pesquisa do Câncer (IARC) classifica o malation como provável carcinogênico em humanos, mas com evidências limitadas a câncer de próstata e linfomas não-Hodgkin, observadas principalmente em trabalhadores rurais diretamente expostos desde 2001. A IARC aponta ainda para o desenvolviento de tumores em roedores expostos ao malation em modelos experimentais, além de causar dano em DNA e ser considerado um desregulador endócrino .

Em 2016, a Associação Brasileira de Saúde Coletiva (ABRASCO) reivindicou a suspensão do uso de malation, tanto como forma de controle do *Aedes aegypt* como em cultura de alimentos. O órgão ainda aponta para a importância de que outras medidas essenciais sejam tomadas para evitar a exposição aos agrotóxicos, como substituição dos produtos por barreiras mecânicas, limpeza e aspiração, telagem em janelas, entre outras medidas.

1.3 Sistema Genital Masculino

O sistema genital masculino é composto por testículos, epidídimos, ductos deferentes, pênis e pelas glândulas sexuais acessórias, tanto em humanos quanto em roedores (Figura 3).

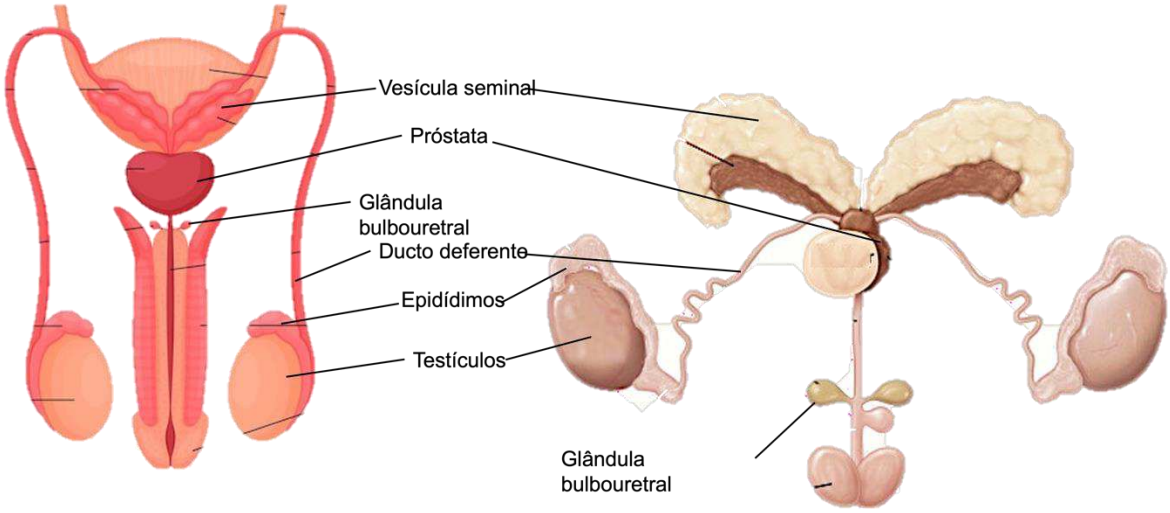

Figura 3. Aspecto macroscópico do sistema genital masculino humano (esquerda) e de rato (direita) adaptado de Knoblaugh e True (2012).

Os testículos são órgãos pares localizados no interior do escroto, revestidos por uma cápsula de tecido conjuntivo denso – a túnica albugínea. Externamente a essa túnica, encontra-se uma camada de peritônio visceral, a túnica vaginal, que também reveste a superfície interior da bolsa escrotal (KOMÁREK, 2000). Morfologicamente, os testículos são compostos por túbulos seminíferos e compartimento intersticial constituído por tecido conjuntivo, responsáveis pela espermatogênese e esteroidogênese, respectivamente (RODRIGUEZ; FAVARETTO, 1999).

Os túbulos seminíferos, as unidades funcionais testiculares, são constituídos por um epitélio germinativo, composto por células somáticas de Sertoli e células da linhagem germinativa (espermatogônias, espermatócitos primários, secundários e espermátides), as quais estão organizadas em camadas concêntricas (FOLEY, 2001). O interstício testicular é composto por tecido conjuntivo, vasos sanguíneos, vasos linfáticos, nervos, macrófagos residentes. Ainda no interstício, estão presentes as células de Leydig, responsáveis pela produção de andrógenos, principalmente a testosterona (RUSSEL, 1990). As células de Leydig possuem retículo endoplasmático liso e mitocôndrias com enzimas associadas à síntese de hormônios estereoides. O citoplasma é eosinofílico e o núcleo arredondado ou alongado.

A testosterona é produzida em resposta ao estímulo exercido pelo o hormônio luteinizante (LH) a receptores de membrana plasmática das células de Leydig (LHR),

estimulando a síntese da proteína reguladora esteroidogênica (StAR), que iniciará uma cascata de eventos na qual o colesterol será convertido em pregnenolona nas mitocôndria e, em seguida, transferido para o retículo endoplasmático liso. As duas maiores classes de enzimas responsáveis por catalisar reações de biossíntese esteroidal são do Citocromo P450 (CYP) e hidroxiesteroide desidrogenase (HSD). Após diversas conversões moleculares por enzimas, a androstenediona é convertida em testosterona pela enzima 17β-HSD (CHAKRABORTY et al., 2021). Na puberdade, a testosterona é crucial para maturação do sistema genital masculino e aparecimento dos caracteres sexuais secundários (RICHMOND; ROGOL, 2007). Em adultos, o andrógeno é responsável pela manutenção da barreira hematotesticular e estimulação da espermatogênese e espermiogênese (MRUK; CHENG, 2015).

A ação biológica de andrógenos é mediada por receptores de andrógenos (AR) que, quando ativados, agem como fatores de transcrição que regulam genes envolvidos em processos biológicos como a diferenciação e maturação sexual, bem como a manutenção da espermatogênese (HEINLEIN; CHANG, 2002). O AR é uma proteína modular organizada em domínios funcionais consistindo em um domínio regulatório N-terminal (NTD), um domínio de ligação ao DNA (DBD), uma pequena região de dobradiça (H) e domínio de ligação a ligante (LBD) (BRINKMANN et al., 1989). Foram identificadas duas isoformas de AR: AR-A não possui os primeiros 187 aminoácidos quando comparado com o AR-B. Enquanto que o DBD e LDB do AR possui regiões altamente conservadas entre as espécies, a evolução da NTD e região de dobradiça H diverge consideravelmente permitindo diferentes controles homeostáticos e de sinalização de AR entre as espécies (WILSON; MCPHAUL, 1994).

A ligação de andrógenos induz alteração conformacional do AR, possibilitando a translocação nuclear, aumento da fosforilação, formação de homodímeros e interação com DNA. Os dímeros de AR formados se ligam a elementos responsivos a andrógenos (AREs) localizados em regiões regulatórias de genes (CLAESSENS et al., 2001), recruta cofatores essenciais e monta a maquinaria transcricional necessária para regular a expressão de genes regulados por andrógenos. Embora o AR funcione normalmente como homodímero, têm sido relatados heterodímeros entre AR e TR4 ou ERα, e que nesses casos resultam em atividade transcricional diminuída do AR. Matsumoto et al. (2013) aponta que desregulação na expressão ou sinalização do AR prejudica o desenvolvimento reprodutivo normal e pode favorecer uma gama de condições patológicas, como a síndrome da insensibilidade a andrógenos, câncer de

próstata e atrofia muscular bulbar espinhal. Como consequência tem-se o prejuízo no processo espermatogênico.

As células de Sertoli são células somáticas presentes nos túbulos seminíferos que se estendem desde a lâminal basal até o lúmen tubular. Possui funções importantes para o processo espermatogênico, fornecendo apoio estrutural para as células da linhagem germinativa e formação da barreira hematotesticular (BHT) através de funções oclusivas (JUNQUEIRA; CARNEIRO, 2005). Além disso, essas células fornecem energia, secretam nutrientes, fatores de crescimento e fatores necessários para o metabolismo das células germinativas (lactato, transferrina e proteína de ligação a andrógenos) (MRUK; CHENG, 2004; SKINNER; ANWAY, 2005). Tais fatores estão envolvidos na transdução dos sinais provenientes do hormônio folículo estimulante (FSH) e da testosterona (WALKER; CHENG, 2005), exercendo participação ativa no processo de espermiação, realizam fagocitose dos corpos resituais e secretam fluidos para o lúmen tubular (FOLEY, 2001; GRISWOLD, 1998).

O processo espermatogênico é organizado em três fases (AMANN, 1986; RUSSEL, 1990), sendo elas: (1) proliferativa, caracterizando-se pela proliferação de células espermatogoniais; (2) meiótica, em que os espermatócitos primários (diploides) passam por meiose I e meiose II, originando espermátides arredondadas (haploides); (3) espermiogênese, em que ocorre processo de citodiferenciação em que espermátides arredondadas atingem a morfologia do gameta masculino. Essa etapa envolve condensação do material genético, formação de acrossoma, reposicionamento das mitocôndrias, perda de citoplasma e formação do flagelo. Tais alterações são cruciais para preparo da morfologia espermática, essencial para a função espermática. A organização mitocondrial adequada fornece energia necessária para motilidade, bem como para adequação da reação acrossômica (ZHANG et al., 2019). Esse processo de citodiferenciação é altamente regulado pela ação da testosterona em receptores de andrógeno (KERR et al., 1992). Após a espermiogênese, as espermátides são tardias e permanecem presas ao epitélio até serem liberadas na luz dos túbulos seminíferos, sendo então denominadas de espermatozoides (CLERMONT, 1972).

A partir da espermiogênese, o tamanho da peça intermediária é um indicador da carga mitocondrial e, portanto, da quantidade de energia disponível para atingir níveis eficazes de motilidade (ANDERSON; DIXSON, 2002). Além disso, Malo et al. (2006) concluem que a motilidade espermática resulta do design combinado entre os

diferentes componentes espermáticos designados durante a espermiogênese. As enzimas proteolíticas organizadas em vesícula denominada acrossoma são outro fator essencial para preparar o espermatozoide para a fusão com o oócito (AITKEN, 1999).

Além dos testículos, os epidídimos são essenciais para garantir a maturação espermática, como a motilidade necessária para fecundação do ovócito II (DACHEUX; DACHEUX, 2014; ROBAIRE; HINTON, 2015). Durante esse processo de maturação, são produzidos baixos níveis de espécies reativas de oxigênio (EROs), importantes no controle dos eventos de fosforilação associados à maturação espermática (AITKEN et al., 1998; AITKEN; BAKER; O'BRYAN, 2004). No entanto, quando produzidas em grandes quantidades ou em falta da capacidade de enzimas e moléculas antioxidantes, pode prejudicar a função espermática através de dano oxidativo na membrana plasmática e em funções como motilidade e exocitose de grânulos acrossômicos. Além disso, o estresse oxidativo em espermatozoide também está associado com indução de dano em DNA de genoma nuclear e mitocondrial (LEWIS; AITKEN, 2001; SAWYER et al., 2003). As enzimas antioxidantes, como SOD, catalase, bem como o sistema glutationa atuam de forma a neutralizar as espécies reativas de oxigênio e diminuir o estresse oxidativo (ZINI et al., 2009).

O ducto deferente conecta o epidídimo à uretra prostática (KOMÁREK, 2000). Em mamíferos, as glândulas sexuais envolvem a glândula seminal, próstata e glândula bulbouretral e estão localizadas ao longo do trajeto que os espermatozoides percorrem desde o ducto deferente até o pênis (HASCHEK; ROUSSEAUX, 1998). A produção de secreções por essas glândulas, de maneira dependente da ação de andrógenos, contribui para a nutrição e suporte dos espermatozoides após a ejaculação (CLEGG et al., 2001; MANN, 1974).

Nos mamíferos, o sêmen é depositado dentro do lúmen vaginal através do órgão copulador, o pênis. O corpo do pênis do rato é formado por dois corpos cavernosos penianos e um corpo cavernoso uretral (CHIASSON, 1988). Histologicamente, o corpo cavernoso é constituído por tecido erétil com auréolas calibrosas, revestidas por uma camada espessa de tecido conjuntivo denso, a túnica albugínea (MURAKAMI; MIZUNO, 1986). A ejaculação do sêmen trata-se de um arco reflexo que sinaliza para a estimulação sensorial peniana, seguida de estimulação motora simpática do músculo liso do sistema genital masculino, bem como estimulação motora somática da musculatura associada aos túbulos desse sistema (STEERS, 1994).

1.4 Desenvolvimento Pós-Natal do Sistema Genital Masculino de Ratos

O período de desenvolvimento após o nascimento pode ser organizado em quatro fases (OJEDA et al. 1980; PICUT et al., 2014): neonatal (dia pós-natal (DPN) 1 – 7), infantil (DPN 8 – 21), juvenil (DPN 22 – 35) e peripuberal (DPN 36 – 65). O animal é considerado adulto quando atinge a maturidade sexual. Essas fases são reguladas especialmente por hormônios (DAMGAARD et al., 2002).

Ao final da gestação, tem-se o início da produção de testosterona, que diminui logo após o nascimento. Durante as fases infantil e juvenil de ratos (DPN 8 – 35) são produzidos os andrógenos primários, incluindo androstenediona, 5-α-androstanediol e diidrotestosterona (PODESTÁ; RIVAROLA; JYUJO, 1974). Entre o DPN 28 e 56, as células de Leydig se diferenciam em células adultas, com pouca ou nenhuma atividade mitótica e alta síntese de testosterona. Além disso, esse período é acompanhado por aumento no número de receptores para LH e diminuição no número de receptores para andrógenos.

Na puberdade, ocorrem mudanças físicas, comportamentais e hormonais que possibilitam a maturação sexual e ganho da capacidade reprodutiva (GOLUB et al., 2008). Essas mudanças resultam de uma cascata de eventos que levam à maturação do eixo hipotalâmico-hipofisário-gonadal. Nesse sentido, a puberdade é marcada pelo aumento da pulsatilidade do hormônio liberador de gonadotrofinas (GnRH), aumento da síntese e secreção de LH e FSH, e consequente aumento da sínetese e secreção de testosterona. Com o pico de testosterona, tem-se ínicio do ciclo reprodutivo da espécie (OJEDA; URBANSKI, 1994).

Biologicamente, a puberdade é definida quando a primeira espermatogênese completa todo o ciclo (KLINEFELTER et al., 1997). No período peripuberal, ocorrem importantes eventos que alteram proliferação e maturação das células germinativas, células de Sertoli, bem como promovem aumento dos níveis de testosterona (O'SHAUGHNESSY, 2015; SHARPE, 2010). Embora esse período seja de grande importância para a maturação sexual, momento em que a espermatogênese e esteroidogênese ainda não estão totalmente estabelecidas (JOHNSON; WELSH; WILKER, 1997), ela tem sido relativamente pouco estudada quanto à intervenção de agentes ambientais potencialmente tóxicos. A exposição a agentes químicos durante os períodos juvenil e peripuberal podem resultar em consequências visíveis mesmo

durantes a puberdade, podendo prejudicar inclusive a integridade sexual na vida adulta.

Tais efeitos estão frequentemente associados com desreguladores endócrinos, os quais interferem na programação do desenvolvimento. Dessa maneira, exposição a tais substâncias durante períodos críticos do desenvolvimento (gestação, lactação e peripuberdade) pode causar prejuízos e comprometer a saúde reprodutiva do indivíduo na vida adulta.

1.5 Sistema Genital Feminino

O sistema genital feminino em humanos é constituído por ovários, útero, tubas uterina e vagina (GRAZIOTTIN; GAMBINI, 2015; HALL; HALL, 2016) (Figura 4A). Similar ao de humanos, o sistema reprodutor feminino de bovinos é constituído por um par de ovários e tubas uterinas, útero e vagina (FRANDSON *et al.*, 2011).Em roedores, é composto também por par de ovários com seus respectivos ovidutos, útero bicornificado e vagina (DIXON *et al.*, 2018; HAMID *et al.*, 2013) (Figura 4B).

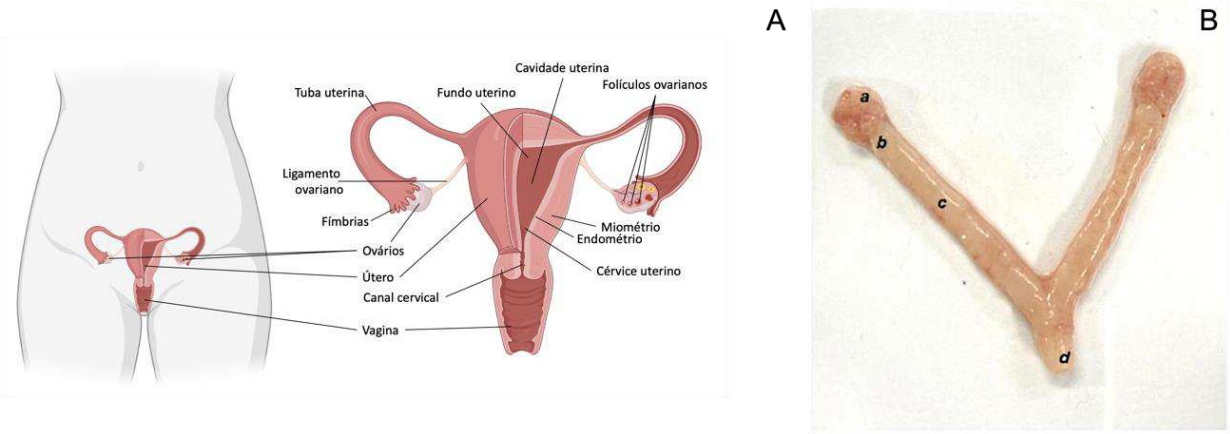

Figura 4. Aspecto mascroscópico do sistema genital feminino. **A** Representação esquemática da organização do sistema genital feminino de humanos. **B** Órgãos do trato genital feminino de ratos. a, ovário; b, tuba uterina; c, corno uterino; d, vagina. Adaptado de SCARTON, 2021).

Tais componentes são essenciais para função desse sistema, que envolve a produção e maturação de ovócitos, bem como a produção dos hormônios sexuais femininos, estrógeno e progesterona. Os ovários são órgãos pares e ovoides

localizados na região pélvica da cavidade abdominal revestidos por tecido conjuntivo denso, formando a túnica albugínea (JUNQUEIRA; CARNEIRO, 2013). Os ovários são organizados nas regiões cortical, mais externa e adjacente onde localizam-se os folículos ovarianos e na região medular, porção mais interna constituída por tecido conjuntivo frouxo que possibilita inervação e vascularização ao órgão (JUNQUEIRA; CARNEIRO, 2013).

As tubas uterinas são órgãos musculares, contendo uma extremidade com abertura para a cavidade peritoneal próximo ao ovário, denominado infundíbulo, e outra extremidade intramural, a qual se insere no óstio uterino (DIXON *et al.*, 2018). Na região do infundíbulo, as tubas uterinas possuem fímbrias que se movimentam ativamente e possibilitam o direcionamento do ovócito para o útero (JUNQUEIRA; CARNEIRO, 2013). Em ratas, o oviduto envolve os ovários em uma estrutura denominada de Bursa ovariana (HAMID *et al.*, 2013). Nas tubas uterinas ocorre então a fertilização do oócito pelo espermatozoide e direcionamento para cavidade uterina (GRAZIOTTIN; GAMBINI, 2015).

O útero humano é organizado em fundo uterino, porção superior e mais dilatada, e o colo uterino, porção estreita e inferior que se estende até a vagina (RENDI *et al.*, 2012), anterior à bexiga. O útero de ratas é constituído por dois cornos fundidos em sua porção caudal, organizados com seu próprio lumen e canal cervical. Essa arquitetura tecidual permite que roedores possam gestar filhotes concomitantemente (DIXON *et al.*, 2018; HAMID *et al.*, 2013). Histologicamente, o útero está organizado em três camadas: endométrio, miométrio e perimétrio.

O endométrio é a camada mais interna do útero, subdividida em camada basal adjacente ao miométrio, constituída por tecido conjuntivo e poucas glândulas, e camada funcional, porção mais interna com maior acúmulo de glândulas uterinas e epitélio luminal. Em humanos, o endométrio é altamente irrigado e sua descamação permite o processo de menstruação. A porção funcional endometrial é a porção que sofre as principais alterações durante o ciclo menstrual (JUNQUEIRA; CARNEIRO, 2013). A segunda camada, miométrio, é constituída por fibras musculares lisas dispostas em diferentes sentidos. A camada mais externa é constituída por tecido conjuntivo e denominada perimétrio (JUNQUEIRA; CARNEIRO, 2013).

A vagina trata-se de órgão fibromuscular, com comprimento de 6 a 12 cm que se estende da vulva até o cérvice uterino. Já em ratas, a vagina apresenta entre 15 a

20 mm de comprimento, possuindo abertura no orifício uretral, assim como em humanos (DIXON *et al.*, 2018; GRAZIOTTIN; GAMBINI, 2015).

A produção de oócito maduro acontece a partir de folículos ovarianos, constituídos pelo ovócito, o gameta feminino, e por células que o envolvem, denominadas células foliculares ou células da granulosa (JUNQUEIRA; CARNEIRO, 2013). De acordo com a organização das células da granulosa, os folículos podem ser classificados em folículos primordiais, primários, secundários, pré-antrais, antrais e folículos de Graaf.

Ao nascimento, as fêmeas possuem folículos primordiais localizados na periferia do córtex ovariano. Os folículos primordiais são caracterizados por um ovócito I circundado por camada única de células foliculares achatadas (BORGEEST *et al.*, 2002; FRANDSON *et al.*, 2011). Estímulos hormonais durante a puberdade possibilitam a diferenciação para folículo primário, contendo um ovócito circundado por camada única de células foliculares cúbicas. O crescimento desse folículo caracteriza o folículo secundário que, possui duas camadas de célula da granulosa cuboides, sendo possível identificar início de formação da zona pelúcida e das tecas. A zona pelúcida é formada a partir de glicoproteínas que envolvem o ovócito, secretadas pelas células da granulosa (RENDI *et al.*, 2012). O desenvolvimento até esse estágio independe de estímulos pelas gonadotrofinas – hormônio luteinizante (LH) e hormônio folículo estimulante (FSH) (FRANDSON *et al.*, 2011; KEZELE; SKINNER, 2003). Os folículos que ainda não possuem antro, sendo eles os primordiais, primário e secundários, são denominados pré-antrais. Folículos antrais são aqueles que apresentam cavidade contendo líquido folicular altamento rico em estógeno. Quando maduros, passam a ser denominados de Graaf, apresentando antro em seu maior tamanho, podendo atingir até 2,5 cm de diâmetro em humanos (JUNQUEIRA; CARNEIRO, 2013).

O FSH e o LH são liberados pela adeno-hipófise em resposta ao estímulo do hormônio liberador de gonadotropinas (GnRH). A liberação de GnRH pode ser modulada por hormônios esteroides e peptídicos dos ovários. O LH estimula a proliferada das células da teca e produção de andrógenos pelas mesmas, enquanto que o FSH estimula a proliferação das células da granulosa e secreção de enzimas conversoras de andrógenos em estrógenos (HAFEZ; HAFEZ, 2004). O FSH, também estimula a secreção de líquido folicular que formará o antro, possibilitando o amadurecimento do folículo. O folículo de Graaf apresenta duas camadas de tecas

bem diferenciadas: camada interna vascularizada com células produtoras de esteroides, e camada externa constituída por tecido conjuntivo (FRANDSON *et al.*, 2011).

O estrogênio produzido pelas células da granulosa atua como agente parácrino promovendo o desenvolvimento folicular. A cada ciclo, de 6 a 12 folículos iniciam seu desenvolvimento mas apenas um chega ao final desse processo. Ao atingir a circulação sistêmica, os estrógenos fazem retroalimentação negativa na adeno-hipófise, diminuindo a secreção de FSH e contribuindo para atresia dos demais folículos (FRANDSON *et al.*, 2011; HAFEZ; HAFEZ, 2004). Além disso, estrógenos sensibilizam os receptores de LH das células tecais, possibilitando aumento da produção de andrógenos por estímulo de LH no momento da ovocitação. As altas concentrações de LH promovem desenvolvimento final do ovócito I e originam o ovócito II e primeiro corpo polar. O ovócito II é liberado durante a ovocitação juntamente com a zona pelúcida e o corpo polar. Os demais folículos regridem, tornando-se atrésicos (RODGERS; IRVING-RODGERS, 2010).

Em resposta às células da granulosa que permanecem no ovário após a ovocitação, há organização das mesmas células lúteas que formarão o corpo lúteo. Após organização dessa estrutura, há parada de síntese de estrógeno e início da produção de progesterona. Além disso, as células lúteas sintetizam moléculas derivadas do ácido araquidônico que promovem a ruptura do folículo (FRANDSON *et al.*, 2011). Durante a gravidez, o corpo lúteo se mantém a produzir progesterona para manutenção do endométrio uterino. Conforme a estrutura de desenvolve, as concentrações de progesterona também aumentam até atingir o nível máximo e as concentrações plasmáticas se estabilizarem (FRANDSON *et al.*, 2011; HAFEZ; HAFEZ, 2004).

Caso não haja prenhez, o corpo lúteo regride através da liberação de PGF_{2a}. As células então entram em apoptose e são substituídas por tecido conjuntivo, formando o *corpo albicans*. Tanto estrogênio quando as inibinas secretadas pelas células da granulosa também contribuem para a atresia folicular. A concentração dessa molécula aumenta com o desenvolvimento folicular (FRANDSON *et al.*, 2011; HAFEZ; HAFEZ, 2004).

Concomitante ao ciclo ovulatório descrito acima, ocorre o ciclo menstrual no útero, caracterizado por uma série de alterações cíclicas no endométrio uterino. Na fase proliferativa tem proliferação das células estromais e glandulares que constituem

o endométrico através da ação dos estrógenos secretados pelos ovários. Já a fase secretora, o endométrio torna-se mais espesso e as glândulas propiciam acúmulo de substâncias secretórias devido aos altos níveis de estrógenos e progesterona secretados pelo corpo lúteo. A finalidade dessa etapa é preparar o útero para implantação do óvulo e permitir a gestação (HALL; HALL, 2016). Caso não haja fertilização do ovócito pelo espermatozoide, e portanto ausência de implantação ma parede uterina, há redução nos níveis hormonais a partir da involução do corpo lúteo e, consequentemente, a menstruação (HALL; HALL, 2016). O resultado do declínio hormonal e da diminuição da chegada de nutrientes é a necrose do endométrio uterino, originando pequenos pontos hemorrágicos que serão expelidos através da vagina no processo denominado menstruação (HALL; HALL, 2016).

Roedores possuem ciclo estral com duração de 4 a 5 dias e organizado em 4 fases: proestro, estro, metaestro e diestro. Dessa forma, as fases estrais das ratas apresentam duração de horas, sendo a diestro mais longa, com duração aproximada de 57 horas (HAMID *et al.*, 2013; OJEDA; URBANSKI, 1994). Durante o ciclo, o epitélio vaginal e cervical passam por alterações significativas que caracterizam as diferentes fases do ciclo (DIXON *et al.*, 2018).

O proestro é caracterizado pela presença de fluido aquoso no útero, sendo observado maiores níveis de estrógeno. No útero, o epitélio é cuboide ou colunar, sendo comumente observada presença de infiltrado inflamatório. Nos ovários, o proestro é caracterizado por degeneração dos corpos lúteos e, ao final dessa fase, as ratas já se encontram receptivas aos machos. Na vagina, pode-se observar figuras mitóticas e pouca degeneração ou descamação (WESTWOOD, 2008).

No início da fase estral, observa-se processo necrótico nas células epiteliais uterinas, com infiltrado leucocitário e diminuição da atividade mitótica dessas células. Nos ovários, são observados corpos lúteos em processo degenerativo com cavidades preenchidas por líquidos e infiltrado basofílico. Nessa fase as fêmeas encontram-se mais receptivas aos machos e permitem a cópula (ANBARKEH *et al.*, 2014; WESTWOOD, 2008). Na vagina, pode-se visualizar a descamação das camadas mucosas e cornificadas, acompanhada de aumento de infiltrado leucocitário (WESTWOOD, 2008).

Em metaestro, há tanto presença de células epiteliais uterinas com alta atividade mitótica quanto em degeneração. Nessa fase, os corpos lúteos ainda possuem resquícios de fluido na cavidade central e, na vagina, há completo

descolamento de camada cornificada e presença de infiltrado inflamatório (WESTWOOD, 2008). Em diestro, o útero encontra-se em seu menor tamanho com poucas mitoses, enquanto que no ovários os corpos lúteos estão em seu maior tamanho (WESTWOOD, 2008). Tanto em metaestro quanto em diestro as fêmeas não encontram-se receptivas aos machos (LOHMILLER et al., 2020). O epitélio vaginal atinge seu ápice em tamanho ao final dessa fase e se mantém até o estro. Nessa fase, há redução de infiltrado leucocitário (DIXON et al., 2018).

1.6 Desenvolvimento Pós-Natal do Sistema Genital Feminino de Ratos

A diferenciação do sistema genital feminino decorre da ausência da expressão da proteína SRY, presente no cromossomo Y. Esse processo tem início no período embrionário, sendo na sétima semana de desenvolvimento em humanos, e entre os dias gestacional 14 e 18 em ratos (DIXON *et al.*, 2018; GRAZIOTTIN; GAMBINI, 2015; SADLER; LANGMAN, 2007).

Logo após o nascimento, ocorrem pulsos de liberação de GnRH pelo hipotálamo tanto em humanos quando em roedores, seguido por período de quiescência, marcado pela ausência deste hormônio em infanto-juvenis (LAFFAN *et al.*, 2017). Assim como em machos, a puberdade em fêmeas também é marcada pela aquisição da capacidade reprodutiva através da maturação do eixo hipotalâmico-hipofisário-gonadal (HHG) (GOLUB *et al.*, 2008; OJEDA *et al.*, 1980). É dita puberdade, o período da vida em que o indivíduo se torna capaz de se reproduzir sexualmente através da maturação dos órgãos genitais, desenvolvimento dos caracteres sexuais secundários, e em humanos, a ocorrência da primeira menstruação. Em mulheres, a puberdade, dada como o período de transição entre a infância e vida adulta, acontece normalmente entre os 11 e 14 anos, caracterizado pelo início de pulsos neuroendócrinos (AGARWAL *et al.*, 2012).

Em ratas, os pulsos geradores de GnRH ocorrem normalmente a partir do DPN 15 através de fatores de crescimentos liberados e a maturação sexual é atingida aproximadamente no DPN 65 (LAFFAN *et al.*, 2017; OJEDA *et al.*, 2006). A liberação pulsada de LH a partir do GnRH estimula então a maturação ovariada e precede o primeiro ciclo estral por aproximadamente 8 ou 9 dias (WESTWOOD, 2008). Por fim,

FSH e LH secretados em resposta à liberação de GnRH estimula a produção de estrógeno e progesterona pelos ovários (HALL; HALL, 2016).

Em ratas, a primeira manifestação física notável do progresso da puberdade é a abertura vaginal, que indica que o eixo HHG foi ativado. No DPN 15, os folículos começam a se desenvolver. No DPN 30, os folículos antrais estão presentes e aptos a serem ovocitados. À medida que os níveis de estrogênios aumentam durante o primeiro proestro, ocorre o desenvolvimento do lúmen vaginal. O primeiro ciclo estral de uma rata acontece aproximadamente uma semana após a abertura do canal vaginal, entre os DPN 30 e 37 (LAFFAN *et al.*, 2017). O ciclo estral de ratas se mostra inicialmente irregular, até que o mesmo atinja um ciclo contendo de 4 a 5 dias. Essa estabilidade do ciclo estral é atingida aproximadamente no DPN 65 (AGARWAL *et al.*, 2012). Vale ressaltar que na maioria das espécies, há um período entre a primeira ovulação e o estabelecimento de ciclos regulares. Mulheres possuem ciclos menstruais monovulatórios que são irregulares até o final da adolescência (GOLDMAN *et al.*, 2007). Quando o ciclo é regular em ratas, as gonadotropinas atuam no proestro com a ovulação acontecendo durante o estro. A detecção dessa fase é feita através de citologia vaginal, sendo observada presença de células queratinizadas (AGARWAL *et al.*, 2012).

1.7 Sistema Genital e o Estresse Oxidativo

O estresse oxidativo é uma condição resultante do desequilíbrio entre espécies oxidantes, como as espécies reativas de oxigênio (EROs) e as espécies antioxidantes capaz de promover uma variedade de desordens celulares . As EROs possuem ao menos um elétron desemparelhado, sendo espécies altamente reativas geradas a partir do metabolismo de oxigênio mitocondrial . As EROs são representadas principalmente pelo oxigênio singlet ($^{-1}O_2$), radical hidroxila (-OH), ânion superóxido (O_2^-), hidroperoxila (HO_2), peróxido de hidrogênio (H_2O_2), hidroperóxidos lipídicos (LOOH) e hidroperóxidos fosfolipídicos (PLOOH) (MELCHIORRI et al., 1996).

Quando em altas concentrações, as EROs podem exercer toxicidade e modificar moléculas biológicas (DAMASCENO et al., 2002), prejudicando a integridade do DNA, além de causar degradação proteica e peroxidação lipídica de membranas (SUN, 1990). A lipoperoxidação de membranas culmina na alteração de sua estrutura e permeabilidade, o que irá comprometer a seletivamente e integridade

da membrana, possibilitando liberação de enzimas lisossomais hidrolíticas para o meio extracelular (MELLO FILHO; HOFFMANN; MENEGHINI, 1984). O ânion superóxido, uma das principais formas de EROs, é formado a partir do oxigênio molecular, pela adição de um elétron de forma espontânea especialmente pela cadeia respiratória mitocondrial (AGARWAL et al., 2014; VERNET et al., 2001).

Esse tipo de reação leva à formação de aldeídos de baixo pelo molecular, como o malondialdeído (MDA), que reage com proteínas plasmáticas e gera outros produtos tóxicos que, frequentemente, induzem a morte celular (HERSHKO, 1989). No entanto, embora frequentemente apontadas por efeito destrutivo nas estruturas celulares, as EROs também estão envolvidas em processos bioquímicos normais, como controle da proliferação e sinalização celular (FINKEL, 1998).

Os mecanismos que visam proteger as células de danos oxidativos por radicais livres são chamados de antioxidantes. Os elementos antioxidantes podem ser classificados em não enzimáticos, como as vitaminas A e C, ou enzimáticos, como a superóxido dismutase (SOD) e catalase (DAMASCENO et al., 2002; NAZIROGLU, 2003).

Durante o desenvolvimento e maturação espermática, os espermatozoides expressam uma série de enzimas antioxidantes que atuam até atingir o trato genital feminino. Por outro lado, as EROs estão envolvidas no ganho de maturação espermática que possibilita a reação acrossômica, bem como na capacitação desse gameta no sistema genital feminino (GRIVEAU; RENARD; LANNOU, 1995). A presença de EROs nesses eventos apontam para o papel fisiológico do estresse oxidativo (LEWIS; AITKEN, 2001).

No entanto, a geração excessiva de EROs pelos espermatozoides pode prejudicar a função dessas células, além de danificar o DNA do gameta (AITKEN, 1999; SHARMA, RAKESH; AGARWAL, 1996). Estudos mostram que o aumento dos níveis de EROs está relacionado com a diminuição da motilidade espermática, prejuízo na espermatogênese ou até mesmo apoptose ativada por dano em DNA (PASQUALOTTO et al., 2001; RITCHIE; KO, 2021). Assim, a diminuição na capacidade de fertilização, o aumento de riscos de abortos espontâneos e anomalias genéticas estão diretamente relacionadas com o aumento dos níveis de estresse oxidativo em espermatozoides (GHARAGOZLOO; AITKEN, 2011).

Assim como em machos, as EROs e os antioxidantes desempenham papel importante em processos fisiológicos do sistema genital feminino. A finalização da

meiose I no ovócito dominante é estimulada por EROS, enquanto que a progressão da meiose II é estimulada por antioxidantes (AGARWAL *et al.*, 2012). O aumento do metabolismo celular para produção de hormônios via citocromo P450 também resulta na formação de EROs, as quais serão importantes inclusive para o processo de ovocitação (RUDER *et al.*, 2009). Enquanto as EROs estimulam a apoptose e atresia folicular, GSH e FSH estimulam o crescimento folicular. O aumento de estrógeno também favorece o aumento da enzima antioxidante catalase e evita a apoptose (BEHRMAN *et al.*, 2001).

O desbalanço entre moléculas oxidantes e antioxidante devido ao aumento de EROs favorece a depleção de ATP, dano tecidual através da inflamação e necrose, e apresentar potencial mutagênico através de danos cumulativos ao DNA (BEHRMAN *et al.*, 2001). Distúrbios envolvendo o sistema genital feminino, como endometriose (MIER-CABRERA *et al.*, 2010) e síndrome do ovário policístico (PALACIO *et al.*, 2006), estão diretamente relacionados com a indução de estresse oxidativo. A atividade das enzimas SOD associadas a cobre ou zinco aumenta no corpo lúteo e diminui na fase de regressão. Além disso, a atividade da mesma aumenta em paralelo com o aumento da produção de progesterona. Por outro lado, a fase de regressão é marcada pelo aumento de EROs e diminuição de SOD (BEHRMAN *et al.*, 2001).

1.8 Fatores Moleculares Relacionados ao Sistema Genital

A expressão de genes de receptores de andrógenos (AR) e sua ativação por andrógenos são cruciais para a regulação, estabelecimento e manutenção da espermatogênese e esteroidogênese (COLLINS e CHANG, 2002). Após ligação entre testosterona e AR, há ativação de vias de sinalização para sobrevivência e maturação celular (TSAI et al., 2006). Tanto as células de Sertoli quanto as células de Leydig expressam o gene AR (ZHOU et al., 2002) e a atuação da testosterona sobre esses receptores é necessária para a espermatogênese mediada por essas células (O'HARA e SMITH, 2015). A biossíntese de testosterona, denominada esteroidogênese, é o processo em que o colesterol é convertido em hormônios esteroides envolvendo proteínas transportadoras, enzimas e cofatores. A maioria das enzimas esteroidogênicas são formas do citocromo P450 ou são hidroxiesteroide desidrogenases. Os genes 17βHSD expressam as enzimas 17β-hidroxiesteroide

desidrogenases, envolvidas na conversão final de androstenediona em testosterona (MILLER, 2011), mostrando-se portanto crucial para a produção final desse andrógeno essencial para a espermatogênese.

Assim como no sistema genital masculino, o crescimento e desenvolvimento dos folículos ovarianos também é mediado por uma série de fatores de crescimento ou fatores inibitórias que desempenham ação nos ovários (OKTEM e URMAN, 2010). A expressão de receptores de estrógeno (ER) nas células das granulosa se mostra essencial para o desenvolvimento folicular, maturação oocitária e processo de esteroidogênese (CHAKRAVARTHI et al., 2021). A expressão do gene ER-α nos ovários possui papel de ativador da transcrição gênica após estímulo por estrógeno e consequentemente da proliferação celular (ZHOU et al., 2005; AHLBORY-DIEKER et al., 2009). Já no útero, a expressão de ER-α é necessária para manutenção do epitélio uterino, sinalização de inibição de vias apoptóticas e garante as respostas adequadas do epitélio ao longo do ciclo uterino (WINUTHAYANON et al., 2010). Por outro lado, a superexpressão desse gene durante a janela de implantação tem sido relacionada com infertilidade (LESSEY et al., 2006; DOROSTGHOAL et al., 2018). Em contrapartida, o ER-β é considerado um gene supressor tumoral devido a seu papel inibitório na transcrição gênica em tecidos mamários e prostáticos (FIXEMER et al., 2003; PARK et al., 2003).

Outro gene que tem sido citado por estimular a proliferação celular é o da β-catenina através do produto de sua transcrição (GUMBINER, 1995). A superexpressão do gene β-catenina tem sido relacionada com diversos tipos de tumores, incluindo câncer ovariano e uterino (YANG et al., 2003; AREND et al., 2013; LUSBY et al., 2013). Além da proliferação celular, são necessários fatores que sinalizem para a sobrevivência celular.

O Bcl-2 é considerado um agente anti-apoptótico cuja ação impede as vias de sinalização para a morte celular programada e possibilita a sobrevivência celular (VAUX et al., 1992; HENGARTNER et al., 1994; HOCKENBERY et al., 1990). No útero, esse gene pode ser expresso pelas células epiteliais glandulares, estromais e musculares lisas. A regulação de sua expressão também ocorre através de hormônios (OTSUKI et al., 1994), especialmente por estímulo de progesterona (MATSUO et al., 1997). Enquanto a diminuição da expressão de Bcl-2 está relacionada com o aumento de corpos apoptóticos nos tecidos, caracterizando sua função anti-apoptótica (OTSUKI et al., 1994), a superexpressão de Bcl-2 possui correlação com diferentes

tumores uterinos (MATSUO et al., 1997; NAKAMURA et al., 1997; TJALMA et al., 1998). Assim como o Bcl-2, o gene Slug também apresenta ação anti-apoptótica (MAJI et al., 2018) e alterações nos seus níveis de expressão também tem sido relacionado a prejuízos à fertilidade feminina (DU et al., 2009; KOLER et al., 2009). Já o gene FGF-2 está relacionado com o desenvolvimento e diferenciação dos folículos ovarianos primordiais em primários (OKTEM e URMAN, 2010).

Em contrapartida, o gene FOXO desempenha importante papel na manutenção da reserva folicular ovariana, desempenhando função inibitória sobre o desenvolvimento dos folículos ovarianos, controlando o crescimento ovocitário (JOHN et al., 2008) e impedindo a progressão do ciclo celular (UHLENHAUT e TREIER, 2011). A ativação de FOXO pode sinalizar para a via de morte celular por apoptose nos folículos, prejudicando a integridade mitocondrial e ativação da via intrínseca da apoptose (LIU et al., 2009). Já no útero, a expressão de FOXO tem se mostrado importante para a manutenção da integridade tecidual e ocorrência da implantação(VASQUEZ et al., 2018). Por fim, o gene TP53, considerado o guardião do genoma, desempenha papel de supressor tumoral, induzindo a parada de ciclo celular, reparo de DNA e, caso necessário, a apoptose da célula (GUIMARAES e HAINAUT, 2002). Por isso, o gene TP53 é desempenhar papel de protejar a célula contra a tumorigênese (TAYLOR et al., 2006).

1.9 Malation e Função Reprodutiva

Antes de estudos experimentais que evidenciassem melhor os efeitos do malation sobre a saúde reprodutiva, a Agência para Substâncias Tóxicas e Registro de Doenças dos EUA (ATSDR) declarou que não havia evidências de toxicidade do composto ao sistema genital masculino (ATSDR, 2003).

Espinoza-Navarro e Bustos-Obregón (2014) mostraram que a exposição de ratos machos adultos ao malation (170 mg/kg) durante 13 dias prejudicou pesos testicular e epididimário, a contagem espermática e levou ao aumento de espermatozoides anormais. Além disso, a exposição de ratos machos adultos ao malation (54 mg/kg) reduziu concentrações plasmáticas de LH, FSH e testosterona (GENG et al. 2015).

Estudos evidenciam que a exposição aguda (500 mg/kg, DPN 45-48) ou crônica (200 mg/kg DPN 21-51) ao malation prejudicou a integridade testicular, níveis hormonais e perfil oxidativo em ratos expostos ao composto (SLIMEN et al. 2014; SELMI et al. 2015). O mesmo foi observado em relação à motilidade, viabilidade, morfologia e contagem espermática. Buscando utilizar doses menores do composto do que disponível na literatura, estudo de Erthal et al. (2020) mostrou que a exposição ao malation durante os períodos juvenil e peripuberal (DPN 25-64) prejudicou também a integridade testicular, níveis hormonais e perfil oxidativo de ratos. Também foram observados danos em relação à morfologia e motilidade espermática (ERTHAL et al., 2020 a; ERTHAL et al., 2020 b).

Embora haja estudos que mostrem prejuízo testicular e de qualidade espermática após exposição experimental ao malation, nenhum deles avalia a expressão de genes envolvidos na produção e ação de andrógenos em testículos. Não obstante, nenhum estudo verificou a função espermática através da avaliação da integridade de acrossomos e da bainha mitocondrial de espermatozoides, bem como o perfil oxidativo de espermatozoides de animais expostos ao malation.

Em relação ao sistema genital feminino, estudos utilizando modelo de exposição ao malation *in vivo* são muito mais escassos. Koç *et al.* (2009) mostraram que a exposição de ratas adultas ao composto (11 mg/kg ou 33 mg/kg) prejudicou a integridade do tecido ovariano, levando ao aumento de folículos atrésicos, diminuição de folículos e do peso do órgão. Os autores apontam ainda que esses danos foram ocasionados através da geração de radicais livres. No entanto, o estudo apresenta os resultados de maneira qualitativa apenas.

Em modelo agudo, foi relatado que exposição única de ratas adultas ao malation (100 mg/kg) via gavage comprometeu o tecido ovariano através da indução de congestão vascular, edema intersticial e degeneração folicular. Além disso, o modelo mostrou que a exposição aguda diminuiu a atividade da enzima SOD, aumentou os níveis de marcadores para a apoptose, embora não tenha sido alterado níveis de MDA (OZSOY *et al.*, 2016).

Já em ratas adultas expostas ao malation (100 mg/kg via intragástrica) durante 2 meses, foi observada redução em número e tamanho de folículos ovarianos associado ao aumento do número de folículos atrésicos. Foi observado ainda aumento de marcadores pró-apoptóticos e diminuição de marcadores para proliferação celular (MADIHA *et al.*, 2011).

Estudos tem mostrado que o estresse oxidativo induzido por malation (50 mg/kg; 2 semanas; via intraperitoneal) desempenha papel importante na indução de dano ao DNA e membrana plasmática, refletindo no aumento dos níveis de MDA e redução de GSH (ARAB *et al.*, 2018). A exposição ao malation (30 mg/kg, 35 dias) também diminuiu a atividade das enzimas antioxidantes SOD e catalase (YONG *et al.*, 2021). Além disso, a apoptose decorrente do estresse oxidativo mostrou prejuízo inclusive para a manutenção do ciclo estral dessas ratas.

Estudos também mostraram que folículos antrais provenientes de caprinos expostos *in vitro* ao malation (10 e 100 nM; 4h, 6h e 8h) apresentaram prejuízo na integridade folicular, sendo observada perda da integridade de membrana, presença de vacúolos nas células da granulosa e picnose. Assim como nos modelos *in vivo*, o estudo apresentou o potencial pró-apoptótico do malation sobre essas células e a redução da atividade das enzimas antioxidantes, SOD e catalase (BHARDWAJ; SARAF, 2014, 2016).

2 JUSTIFICATIVA

Dentre os problemas de saúde pública a nível nacional, destacam-se as arboviroses. Trata-se de doenças ocasionadas pelos vírus da dengue, zica e chikungunya transmitidas pelo mosquito vetor *Aedes aegypt*. A principal forma de conter o avanço dessas doenças é pelo controle desse mosquito através de inseticidas químicos. Dentre as classes de inseticidas, se destacam os organofosforados, inibidores da acetilcolinesterase que são pulverizados ao longo das cidades e zona rural. Embora o alvo sejam os mosquitos, tais compostos atingem culturas agrícolas e as casas de pessoas que vivem em regiões endêmicas dessas doenças. Seja em ambientais urbanos pelas medidas de combate ao mosquito ou em ambientes rurais através da utilização em meios de cultivo, a população está exposta a organofosforados por via dérmica, inalatória, mas também por via oral através de alimentos contaminados.

Após o *A. aegypt* adquirir resistência à deltametrina, o organofosforado de escolha no Brasil passou a ser o malation. Embora muitas vezes apresentado como seguro, estudos tem mostrado que o malation pode prejudicar a saúde reprodutiva tanto de machos quando de fêmeas. Por outro lado, a grande maioria dos estudos abordam doses de malation que não representam a exposição à qual estamos submetidos, permanecendo incerto quais danos a exposição real de malation pode causar a tais sistemas. Além disso, não existem estudos que avaliem o desenvolvimento pós-natal de animais submetidos à exposição de malation durante os períodos juvenil e peripuberal, levando em conta expressão gênica, qualidade de gametas e níveis de estresse oxidativo.

O período supracitado mostra-se crítico para o desenvolvimento do sistema genital, tanto feminino quanto masculino, uma vez que ocorre a maturação do eixo hipotalâmico hipofisário-gonadal, essencial para desenvolvimento da função e caracteres sexuais secundários. Deve-se considerar que juvenis e adolescentes de áreas endêmicas estão expostos ao inseticida malation continuamente e de forma não intencional. Dessa forma, o presente estudo possui grande relevância ao avaliar os efeitos da exposição ao malation em diferentes e baixas doses sobre o sistema genital feminino e masculino de ratos durante a juventude e peripuberdade. Além disso, será avaliado os mecanismos pelo qual o malation prejudica a integridade folicular utilizando-se modelo de explante tecidual *in vitro*. A partir dos resultados obtidos,

pretende-se contribuir com melhor compreensão da ação de baixas doses de malation sobre o desenvolvimento pós-natal do sistema genital e sua possível relação com diminuições nos níveis de fertilidade.

3 OBJETIVOS

3.1 GERAL

Diante da falta de informações específicas na literatura e da relevância clínica, sociale política do assunto, o objetivo do presente trabalho foi avaliar se a exposição a baixas doses de malation pode comprometer a qualidade de espermatozoides, de folículos ovarianos e o desenvolvimento do sistema genital feminino, bem como os mecanismos envolvidos no dano.

3.2 ESPECÍFICOS

- Determinar os mecanismos pelos quais o malation pode prejudicar a síntese e ação da testosterona em testículos
- Avaliar a função espermática por meio de parâmetros da integridade acrossômica e da bainha mitocondrial
- Verificar a correlação entre a síntese de testosterona por células de Leydig TM3 com o perfil oxidativo
- Determinar as concentrações de citocinas pelas Leydig TM3 expostas ao malation
- Verificar se existe alterações na expressão gênica em testículos após exposição ao malation
- Analisar a fisiopatologia ovariana e uterina após exposição ao malation através de dosagens hormonais, avaliação de integridade e morfometria tecidual e identificação de desenvolvimento folicular
- Contribuir com dados sobre os possíveis mecanismos de ação do malation no sistema genital feminino através de identificação de marcadores de estresse oxidativo e expressão gênica

4 ARTIGO 1

Downregulation of AR and 17-ß-HSD testicular gene expression compromises sperm quality in rats exposed to low doses of malathion during juvenile and peripubertal periods

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Downregulation of AR and 17-ß-HSD testicular gene expression compromises sperm quality in rats exposed to low doses of malathion during juvenile and peripubertal periods

Rafaela Pires Erthal[1,2]*, Gláucia Eloisa Munhoz de Lion Siervo[1,2], Giovanna Fachetti Frigoli [1], Tiago Henrique Zaninelli[2], Waldiceu Aparecido Verri Jr[2], Glaura Scantamburlo Alves Fernandes[1]

1 – Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

2 – Department of General Pathology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

Malathion impairs testicular and sperm development

***Corresponding author**
Rafaela Pires Erthal
Tel.: +55 43 999131971
E-mail address: rafaela.erthal@uel.br (R. P. Erthal)

Abstract

Malathion is an insecticide used to control arboviruses and agricultural pests. Since adolescents exposed to this insecticide are more vulnerable because they are in the critical period of post-natal sexual development, the aim of this study was to evaluate the damage malation can cause to sperm function and its respective mechanisms when exposed during postnatal sexual development. For so, 24 male Wistar rats (PND 25) were distributed in 3 experimental groups and daily treated for 40 days: control group (saline 0,9%), 10 mg/kg (M10 group) or 50 mg/kg (M50 group) of malathion. At PND 65, rats were anaesthetized and euthanized. Testicles were collected for evaluation of gene expression. Sperm cells from epididymis for evaluation of oxidative profile or spermatic function. Data showed that the lower dose of Malathion downregulated gene expression of androgen receptors (AR) and testosterone converter enzyme 17-b-HSD in testis. Compromise in acrosomal integrity of sperm cells was observed in the M50 group, but not in M10 group. Mitochondrial activity was not impaired by exposure. Finally, as though no alterations in MDA and GSH levels were observed, malathion, in both doses, increased antioxidant enzyme catalase activity, and, at a higher dose, superoxide dismutase (SOD) activity. From this data, this study showed that doses of malathion considered harmless so far impaired spermatic quality and function through testicular downregulation of AR and 17-b-HSD and through oxidative stress in sperm cells. Considering adolescents exposure to malathion, our aim is to draw attention to human exposure to this pesticide per environmental contamination.

Keywords: sperm, gene expression, malathion, antioxidants, postnatal development

1. Introduction

Erradication of the *Aedes aegypt* mosquito is one of the major measures taken aiming the control of arboviruses spread. A compound used worldwide for this purpose and in agriculture is an insecticide known as Malathion [1]. This organophosphate can be broadly found environmentally [2] and it is known to have low toxicity in humans when in comparison to other compounds in this class [3].

The World Health Organization (WHO) points that continuous use of malathion, especially in epidemic periods of diseases caused by the Dengue and Zika viruses, has implications [4]. For environmental control, it was stipulated by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) that the maximum residue quantity allowed in food crops is of 8 ppm of malathion to avoid possible complications due to exposition to this compound. For adult rats, the LD50 of malathion is 5400 mg/kg and the NOAEL for the under development reproductive system is presented as 130 mg/kg [5]. Previous studies from our group showed that exposition to low doses of malathion, when compared to the NOAEL and LD50 for rats, were prejudicial to the morphological post-natal development of testis and epididymis [6–7].

Children and teenagers are constantly growing and developing during periods of mosquito eradication and, therefore, exposed though contaminated food consumption or even through malathion pulverization in cities [2]. During development, the organism is plastic and gene expression and cell signaling pathways are more susceptible to external agents [8]. The hypothesis that influences by environmental factors during early life favors the development of diseases in later life is called the Developmental Origins of Health and Disease (DOHaD). It has been proposed from the DOHAD that the discrepancy between the predicted and the developmental environment can negatively affect an individual's health and increase the disease risk [9].

The juvenile and peripubertal periods are known to be a critical window to the sexual development, in which individuals are highly susceptible to the action of toxic compounds. This susceptibility is due to alterations that occur in this period, involving the production of primary androgens, that in rats occur between PND 8-35 [10] and differentiation of Leydig cells, between PND 28-56 [11].

During the period known as peripuberty, in rats (PND 35-65), occurs the determinizing factor to puberty installation: maturation of the hypothalamic-pituitary-testicular axis and the production of high concentrations of testosterone [12]. Androgens produced in high concentrations act in androgen receptors (AR), that trigger intracellular cascades, stimulating spermatogenesis and hormonal biosynthesis though converter enzymes, such as 17-β-HSD [13]. Given the circumstances, the juvenile and peripubertal periods showed to be more sensitive to the action of toxic agents, insults during these periods can result in temporary or permanent damage to the male reproductive system.

The mature male reproductive system may be able to produce functional sperm cells to fecundate oocytes. For this, the testis may produce viable sperm cells with adequate morphology, involving adequate distribution of mitochondria for mitochondrial sheath formation and enzymatic organization for acrosome formation during spermiogenesis in testis [14]. After sperm formation during testicular spermiogenesis, these cells acquire the sperm capacitation necessary for oocyte fertilization, such as performing the acrosome reaction and motility, dependent on the energy provided by the mitochondrial sheath [14].

Authors emphasize that, in physiological levels, ROS regulate intracellular cascades that enable the hyperactivation, capacitation and acrosomal reaction in sperm cells [15]. However, the quality of human semen is directly related to sufficient levels of antioxidants and low levels or reactive species of oxygen (ROS) [16]. It’s interesting to observe that the destructive role of

intracellular oxidative stress is very well known and recognized, meanwhile the physiological role of this event in spermatic capacitation exists but it's not as well-known.

Studies show that rats exposure to malathion causes organ damage through alterations in the oxidative profile [7–17]. An *in vitro* study showed that goat's testis exposed to malathion for 8 hours (100 ng/mL) impaired antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) activities [18]. However, there are no studies in literature evaluating spermatic oxidative profile of rats exposed to low doses of malathion during the juvenile and peripubertal periods.

Studies addressing the DOHAD hypothesis have shown that exposure to endocrine disrupting agents as environmental contaminants during important periods of development impairs important parameters involved in the establishment of sexual maturation. In addition, most of these studies involving the DOHAD concept assess the exposure of animals during the prenatal/gestational period [19–21].

Little has been evaluated of the sperm functionality and impairment mechanism of malathion exposure during juvenile and peripuberty - critical periods of sexual development. In addition, exposure to toxicants in the postnatal development of the male reproductive system has been little explored from the perspective of DOHAD. On the light of that, the aim and innovation of the present study was to evaluate if exposition to low doses of malathion during the post-natal period could impair the spermatic physiology and the possible mechanisms involved in this impairment.

2. Material and methods

2.1. Animals and experimental conditions

Twenty four juvenile male Wistar rats from different litters at postnatal day 21 (PND21) were supplied by the Animal House of Biological Sciences Centre, State University of Londrina

(CCB - UEL), and were acclimated to the new environment at the Laboratory of Toxicology and Metabolic Dysfunction of Reproduction for 4 days right before the beginning of the experimental period. The animals were kept under recommended conditions at the local animal house. The animals were allocated into polypropylene cages (43 × 30 × 15 cm) (3 animals/cage) with laboratory-grade pine shavings as bedding during the entire experiment. The temperature and lighting were controlled (~ 23°C; 12L, 12D photoperiod, lights switched off at 07:00 pm). Rat chow and filtered tap water were provided *ad libitum*. Animal care and handling procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and with the approval of the Ethics Committee on Animal Use of State University of Londrina (CEUA/UEL protocol number 12305.2016.65).

2.2. Experimental design

The animals were randomly assigned to three experimental groups of 8 animals each: control (C), malathion 10 mg/kg body weight (b.w.) (M10) and malathion 50 mg/kg b.w. (M50). We used malathion at doses lower than the subchronic NOAEL (no observed adverse effect level) dose (130 mg/kg b.w.) for the reproductive system in rats in relation to developmental toxicity [5]. In addition, the average of doses used in the present study represents the dosimetric adjustment [22] of the AOEL dose of 0.03 mg/bw/day in humans (European Commission) with an added security factor of 10 considering intraspecies variability [23]. So, these doses are considered low and relatively safe in relation to these parameters and previous studies [24].

The malathion doses were administered according to Geng et al. (2015) [25], which demonstrated reproductive disorders in Wistar rats that were exposed to 54 mg/kg b.w. malathion during adult life. However, in the current study, the experimental period was modified to PND 25 to 65, to reach the juvenile and peripubertal periods established according

to Ojeda et al. (1980) [26]. The animals were exposed to malathion via oral gavage with 10 or 50 mg/kg b.w. diluted in 0.9% saline as vehicle or were vehicle-treated for the control group. All groups were treated daily for 40 consecutive days.

2.3. Preparation of malathion solution

Malathion (dietil-dimetoxitiofosforiltio; CAS no. 121-75-5; Cheminova) was obtained from Dominus Quimica (Jandaia do Sul, Brazil). The compound was diluted in 0.9% saline daily as vehicle.

2.4. Testis and sperm collection

At the end of the experimental period, the rats were intraperitoneally anaesthetized with a combination of ketamine 75 mg/kg b.w. (Sedomin® 10%, Avellaneda, Argentina) and xylazine 10 mg/kg b.w. (Anasedan®, Paulínia, Brazil), weighed and euthanized via cardiac puncture. The testes were removed and the right testes weights were determined (n=10 rats per group) and used for gene expression by RT-qPCR. Spermatozoa from the tail of epididymis were used for sperm functional analysis (n=06 per group) and evaluation of oxidative stress (n=08 per group).

2.5. Mitochondrial activity

The mitochondrial activity of the sperm (n = 06) was determined as described by Silva et al. (2014) [27] with adaptations. Sperm obtained from the tail of the epididymis were added in microtubes containing 1 mg / mL of 3-30-diaminobenzidine (DAB) dissolved in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.03 mM Na2HPO4, KH2PO4 1.47 mM, pH 7.4) in a 1: 3 (v / v) ratio and incubated at 37 ° C for 1 h in the dark. Smears were prepared under histological slides and fixed with 10% formaldehyde for 10 min. Two hundred cells were

evaluated with a phase-contrast microscope and classified as: DAB-I (stained intermediate piece, indicating that the cells maintain a complete mitochondrial activity or little loss of mitochondrial activity, which may not lead to severe impairment of motility and capacity fertilization); DAB-II (absence of staining in the intermediate part, indicating dead cells or cells that maintain minimal energy production through oxidative phosphorylation).

2.6. Acrosome integrity

Sperm acrosome status was evaluated as described previously by Silva et al. (2014) [27]. Smears were prepared onto microscope slides using fresh sperm suspension (obtained from cauda epididymis) and fixed with methanol (n=6/group). Slides were then stained with 40 μg/mL fluorescein-labeled PNA (FITC-PNA; Sigma- Aldrich, St Louis, MO, USA) in PBS and covered with Fluoromount-G with DAPI (EMS, Hatfield, PA, USA). Two hundred cells per slide were analyzed under a fluorescence Axio Zeiss microscope (Zeiss®, Thornwood, NY) equipped with appropriated excitation/emission filters, and cells were classified as Intact acrosome (intensively bright fluorescence of acrosome cap) and disrupted acrosome (disrupted fluorescence of acrosome cap).

2.7. Oxidative profile of sperm cells

The sperm collected from epididymis tail were homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at 9,500 g for 10 min at 4oC. The protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as a standard [28]. Samples were then normalized to 1 mg protein/mL and used for the following analyzes. The analysis of the oxidative profile were performed through the quantification of lipid peroxidation and other antioxidant substances.

2.7.1 Lipid peroxidation

The LPO was measured to indirectly quantify the peroxides produced. The result reflects the intensity of lipid peroxidation [29]. Measurements were performed using the method of reactive substances to thiobarbituric acid (TBARS) with an absorbance of 535 nm and 572 nm [30] compared to the standard curve for malondialdehyde (MDA), the main by- product of cellular lipid peroxidation. To prepare the test, 50 µL of each normalized sample was pipetted in duplicate in a microplate, followed by the addition of $FeCl_3$ (1M), ascorbic shaked and placed in a water bath at 90º C for 15 minutes. The plate was then cooled to stop the reaction, and then read at 535 and 572 nml. Lipid peroxidation (LPO) was estimated correcting for the amount of protein, and the results are expressed in nmol of TBARS per mg of protein.

2.7.2 Reduced gluthatione

Reduced glutathione (GSH) levels were determined as proposed by Rahman et al. (2007) [31], with some modifications. For this, 5,5-dithiobis (2-nitrobenzoic acid) NBT was used in the sperm homogenate supernatant and evidenced by a yellow color formation. GSH levels were measured at 412 nm and results expressed as micromols/mg protein.

2.7.3 Catalase activity

The enzymatic activity of catalase (CAT) was be determined by the degradation of hydrogen peroxide into oxygen and water. After determining the protein concentration (normalized 1.0 mg/ml in PBS), 297 µl of reaction medium was be placed in a UV4 microplate (in triplicate) at 240nm for 60 seconds [32].

2.7.4 Superoxide dismutase activity

The evaluation of the activity of the enzyme superoxide dismutase (SOD) was performed as described by Senthilkumar et al. (2021) [33] with some changes. The enzyme comes from homogenates normalized to 1mg/ml. A reaction mixture was prepared containing sodium carbonate buffer (50mM, pH 10.2), nitroblue tetrazoilium (NBT) (96 uM) and Triton X-100 (0.6%), which was incubated for 2 minutes with sodium hydrochloride. hydroxylamine (NH2OH·HCl) (20 mM, pH 6.0). The final volume was adjusted to 200 µL. The reaction consists of the quantification of complexes formed by superoxide anions with the addition of NBT and NH2OH·HCl of yellowish color with the reduction of NBT, forming a bluish color read at 560 nm for 2 minutes at intervals of 15 seconds.

2.7.5 Glutathione S-transferase activity

The enzymatic activity of glutathione S-transferase (GST - EC 2.5.1.18) of the sperm was determined through the formation of a thioether from the interaction of GSH with CDNB, the increase in absorbance through the formation of the thioether was monitored at 340 nm (RS: 100 mM potassium phosphate buffer pH 6.5; 1.5 mM GSH; 2 mM CDNB) for 5 minutes at 40 second intervals, as described by Keen et al. (1976) [34]. Values were expressed in µM Thioether formed.min^{-1}.mg $protein^{-1}$.

2.8. Quantitative and real-time polymerase chain reaction (RT-QPCR)

RT-qPCR was performed as previously described by Manchope et al. (2016) [35]. Collected testis samples were homogenized in Trizol reagent and total RNA was extracted using the SV Total RNA Isolation System kit (Promega). The purity of total RNA was measured with a spectrophotometer with the wavelength absorption ratio (260/280 nm) being between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA, and qPCR were carried

out using GoTaq® 2-Step RT-qPCR System (Promega) following the manufacturer's instructions.

All reactions were performed in triplicate using the following cycling conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. qPCR was performed in a LightCycler Nano Instrument thermocycler (Roche, Mississauga, ON, USA) by sequence detection system using Platinum SYBR Green RTqPCR SuperMix UDG (Invitrogen, USA). β-actin mRNA levels were used as a control method to assess tissue integrity in all samples. The relative gene expression was measured using the comparative 2−(ΔΔCq) method.

The primers used were to evaluate the expression of genes for AR, sense: *5'-GGAGAACTCTTCAGAGCAAG-3'*, antisense: *5'-AGCTGAGTCATCCTGATCTG-3'*; and 17-b-HSD, sense: *5'-AATATGTCACGATTGGAGCTGA-3*', antisense: *5'-AAGGAATCAGGTTCAGAATTATCG-3'* being respectively involved in the action and synthesis of testosterone. The primers used for β-actin gene were: sense: *5'-GCCATGTACGTAGCCATCCA -3'*, antisense: *5'- GAACCGCTCATTGCCGATAG-3'*

2.9. Statistical analysis

One-way analysis of variance (ANOVA) with post hoc Dunnett's test or the non-parametric Kruskal–Wallis test with the Dunn's post hoc test was used to compare the results between the experimental groups. The Barttlett`s test was performed to evaluate the variance among the experimental groups and the normal distribution was compared using Shapiro-Wilk test. Data are presented as the mean ± s.e.m. Differences were considered significant when $p < 0.05$. The statistical analyses and graph design for the results were performed using GraphPad Prism for Windows (version 7.01 – GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Sperm function: mitochrondrial activity and acrosome integrity

Although the mitochondrial activity was not affected by the exposure to both doses of malathion, the major dose of the compound was sufficient to increase the percentage of non-intact acrosome of sperm form rats (Table 1).

3.2. Oxidative profile of sperm cells

The biomarkers of oxidative stress are shown in Fig. 1. The MDA levels were not altered by the different doses of malathion, as well the antioxidant GSH or the activity of the enzyme GST. On the other hand, the activity of CAT enzyme was increased in rats exposed to malathion 10 or 50 mg/kg. The same occurred with SOD enzyme, which increased in group M50, but not at M10 in relation to control group.

3.3. Quantitative and real-time polymerase chain reaction (RT-qPCR) in testis

The Fig. 2 shows that the minor dose of malathion decreased the gene expression of both AR and 17-ß-HSD genes in testicle rats exposed during juvenile and peripubertal periods. The M50 group did not differ from control group.

4. Discussion

The present article brings as novelty the possible mechanisms involved in spermatic quality alteration after exposure to malathion during the juvenile and peripubertal periods. Our group's previous studies showed that exposure to low doses of malathion during the juvenile and peripubertal periods were sufficient to alter testicular integrity and spermatic [7] and epididymal [6] morphology. To observe spermatic physiology is as important as its morphology to infer the individual's fertility potential. On the light of that, the decrease in acrosomal integrity reported in this study is crucial for spermatic function and oocyte fertilization.

Defects in the process of spermiogenesis after exposure to toxic agents can be related to low sperm counts, increased proportion of abnormal sperm, reduced acrosome integrity, and impaired motility [36]. Corroborating O'Donnel (2015) [36], previous studies have highlighted the impairment of spermiogenesis following exposure to low doses of malathion [6–7], manifested in morphological and sperm motility alterations. The same impairment in this process is evidenced in the present study after a decrease in acrosomal integrity was observed in rats exposed to low doses of malathion during the juvenile and peripubertal periods.

The acrosome is an organelle formed during spermatogenesis situated in the apical region of the spermatozoon, composed of enzymes from lysosomes, peroxisomes and even from the cytoplasm [37–38]. Its protein components are synthesized even before the development of the male gamete. The formation of this organelle is a complex and highly regulated phenomenon compared to other organelles [39–40].

The liberation of acrosome enzymes after the sperm cell binds itself to the oocyte's zona pellucida is known as acrosomal reaction and the result of this is the creation of pores in the oocytes membrane, necessary for penetration of extracellular coat of oocyte [41]. In this process, ROS have been pointed as a facilitators via phosphorylation of tyrosine proteins that allow calcium influx and consequential fusion of the sperm cell to the oocyte – fertilization [42–43].

In this study, we did not observe alterations in the sperm peroxidation levels between experimental groups. On the other hand, this does not mean that there was no oxidative stress caused by malathion in the sperm cells given that low doses of this pesticide were responsible for increasing the activity of antioxidant enzymes SOD and CAT, altering the antioxidant profile as a way of compensating for a disturbance by oxidative stress in these cells. The goal of this molecules and antioxidant enzymes is to neutralize ROS, avoiding oxidative damage [44] in the sperm cell.

Corroborating our results, Kocabaş et al. (2018) [45] showed that sperm cells exposure to malathion in an *in vitro* model (75, 100 e 125 ug/l) increased antioxidant enzyme CAT activity and reduced SOD's, even though MDA and GSH levels were unaltered. Given so, we confirm that malathion alters the oxidative status of sperm cells regardless of the model used.

Reforcing malathion's destructive role to spermatic function, our group previous study showed that exposition to low doses of malathion during peripuberty compromised spermatic motility [6]. However, due to new data, we concluded that this motility alteration was not a result of alterations in spermatic mitochondrial sheath, once this structure was unaltered after exposure to this insecticide.

Once sperm cells are produced in the testis, some of the spermatic impairment observed in our group's previous studies, such as in spermatic production and morphology [7], can be justified by the alterations in the hormone synthesis and signaling pathway observed in our study, evidenced by downregulation of AR receptors and of 17-β-HSD enzyme after rats exposure to malathion.

Previous studies that showed impairment in testosterone production after malathion exposure [7, 46–47] are now justified by downregulation of 17-β-HSD observed in this study. This gene is related to the final stages of the steroidal hormone synthesis catalytic reaction, being the produced enzyme responsible for catalyzing the conversion of androstenedione in testosterone [48].

The binding between androgens and AR and its regulations are crucial to the regulations and establishment of spermatogenesis [49]. Therefore, a disturbance in the expression of this receptor compromises spermatogenesis performance. Qiu et al. (2013) [50] reported that doses of bisphenol A (BPA), another toxic compound broadly found environmentally, beneath NOAEL, were enough to downregulate AR and 17-β-HSD expression, consequently compromising spermatogenesis and spermatic quality. The same correlation was made in the

present study. It is interesting that the reduction on the number of Sertoli cells observed in a previous study using the same experimental model [7] is also directly related to the downregulation of AR reported in the present study. Impairment to Sertoli cels through this via mcompromises the integrity of the seminiferous epithelium, spermatogenesis and, consequently, spermatic quality [51].

A point to be highlighted is that genetic expression was only downregulated in M10 group, the lowest experimental dose, when in comparison to control group. Interestingly, another previous study showed that just the lowest dose (10 mg/kg) of malathion compromised sperm motility [6], another important parameter for spermatic function evaluation. This is a curious fact once the highest dose used in the present study did not cause the same alterations. On another hand, it is not the first nor the last time in which this is reported in literature [52]. The toxic agent Bisphenol S is one of the most accused to impair spermatogenesis and sperm quality in lower or very low doses [52]. Darghouthi et al. (2022) [53] reported that low doses of Bisphenol S impaired spermatic quality, altered the conformation of StaR protein involved in the production cascade of steroidal hormones, and impaired antioxidant species with oxidative profile alterations in rats. The same occurs with Dichlorodiphenyltrichloroethane (DDT), that in lower doses alters intermediate proteins involved in the production of steroidal hormones [54]. In the literature, however, there were no studies the effects and mechanisms of damage after exposure to low doses of malathion on sperm quality. Thus, a novelty is presented about this insecticide widely used mainly in underdeveloped tropical countries.

As the aforementioned studies, our study shows that malathion in low doses induces alterations acting as an endocrine disruptor, downregulating the expression of the testosterone converter enzyme, 17-b-HSD, and AR. Chaturvedi et al. (2010) [55] emphasizes that endocrine disruptors involve not only hormone-like compounds, but also those capable of impairing the synthesis and/or modulators of androgen receptors.

In the present study, we address a critical period for tissue reprogramming during sexual development through the DOHAD hypothesis [56]. Although adaptations are beneficial to the body, when the individual is exposed to a different environment than anticipated during part of development, there is increased risk of disease [57]. Moreover, according to this hypothesis, the early life environment has a prominent influence on the individual's health in later life.

This exposure involves the introduction of chemicals and pollutants that require the body to adapt. In this context, studies confirm that the influence of these toxic agents into epigenetics during critical periods of development are able to modulate hormone signaling through period gene plasticity, corroborating with our data [57].

We highlight that this is the first study to evaluate spermatic function through spermatic integrity parameters, mitochondrial activity, oxidative profile in sperm cells and genetic expression in the testis after rats exposed to low doses of malathion, especially during the juvenile and peripubertal periods. Our data indicates that animals exposed to malathion during critical periods of sexual development might have their reproductive health compromised even during adulthood.

Although population studies are needed to evaluate the effects of malathion and apply the newly formed knowledge into the clinical practice, the whole approach through DOHAD achieved by the present experimental model allows establishing causal associations through the mechanisms addressed and to illuminate new strategies for the prevention, prognosis and intervention by idiopathic infertility [58].

5. Conclusion

The present study showed that low doses of malathion considered to be inoffensive are capable of impair spermatic quality and function through downregulation of testicular genic

expression of AR e 17-b-HSD, and damage to the spermatic antioxidant profile during these critical periods of development. Considering the presence of malathion in the environment and exposure of children and teenagers to this pesticide, our goal is to increase awareness to exposition of human beings to this compound through environmental contact.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Ethical standards.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals and has been approved by the Ethics Committee on Animal Use of State University of Londrina (CEUA/UEL protocol number 12305.2016.65).

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Table legends

Table 1. Effects of juvenile and peripubertal exposure to low doses of malathion on sperm functional parameters

Data are presented as the mean ± s.e.m. 1One-way ANOVA test with a posteriori Dunnett's test. 2Kruskal-Wallis test with the post hoc Dunn's test. *$p<0.05$. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion. DAB I – total mitochondrial active; DAB II – mitochondrial partially

Figure legends

Figure 1 – Oxidative profile in the sperm cells from rats exposed to vehicle or malathion at 10 mg/kg or 50 mg/kg. (A) Lipid peroxidation assay. (B) Reduced glutathione (GSH) levels. (C) catalase (CAT), (D) superoxide dismutase (SOD) and (E) glutathione-S-transferase (GST) activity, in the supernatant of the sperm cells. ANOVA test followed by Dunnett's test. Data are represented as the mean ± s.e.m. ***$p < 0.001$ compared with control. *$p < 0.05$ compared with control. M10 - rats treated with 10 mg kg^{-1} malathion; M50 - rats treated with 50 mg kg^{-1} malathion.

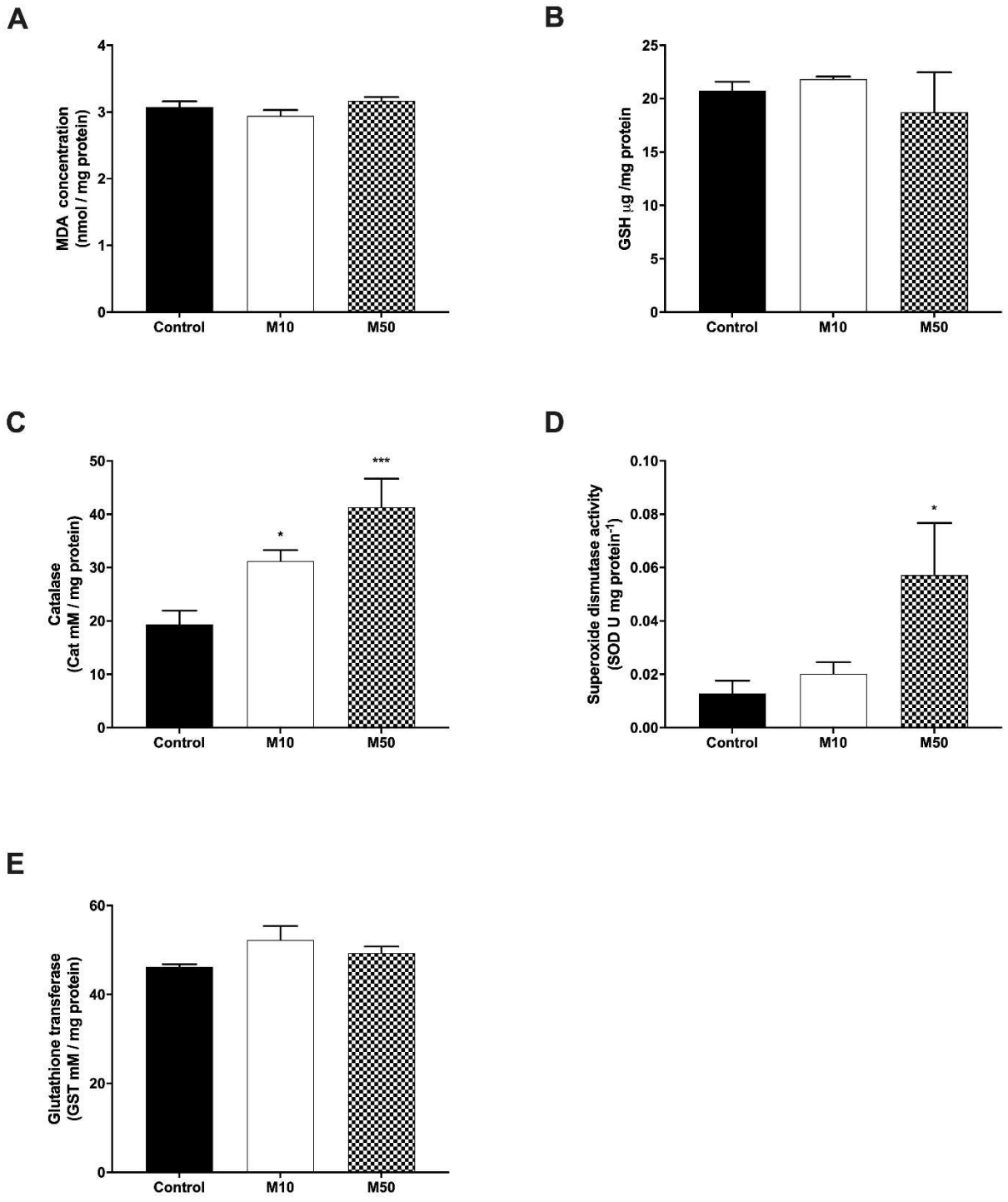
Figure 2 – Gene expression of (A) androgen receptor and (B) 17-b-HSD in testis from animals exposed to vehicle or malathion at 10 mg/kg or 50 mg/kg.

Values are expressed as the mean ± s.e.m. *$p < 0.05$. **$p<0.01$. One-way ANOVA test, with post hoc Dunnett's test. M10 - rats treated with 10 mg kg^{-1} malathion; M50 - rats treated with 50 mg kg^{-1} malathion.

Table 1. Effects of juvenile and peripubertal exposure to low doses of malathion on sperm functional parameters

	Experimental groups		
Parameters	**Control (n=10 animals)**	**M10 (n=10 animals)**	**M50 (n=10 animals)**
Mitocondrial activity (%)			
[1]DAB I	88.6 ± 2.5	89.8 ± 1.6	86.2 ± 5.3
[1]DAB II	9.9 ± 2.5	9.5 ± 1.5	8.2 ± 2.6
[2]DAB III	1.5 ± 0.5	0.5 ± 0.5	5.6 ± 5.1
Acrosomal integrity (%)			
[1]Intact acrosome	95.2 ± 0.7	93.5 ± 1.1	91.8 ± 0.7 *
[1]Non-intact acrosome	5.8 ± 0.7	6.5 ± 1.1	8.2 ± 0.7 *

Data are presented as the mean ± s.e.m. 1One-way ANOVA test with a posteriori Dunnett's test. 2Kruskal-Wallis test with the post hoc Dunn's test. *$p<0.05$. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion. DAB I – total mitochondrial active; DAB II – mitochondrial partially



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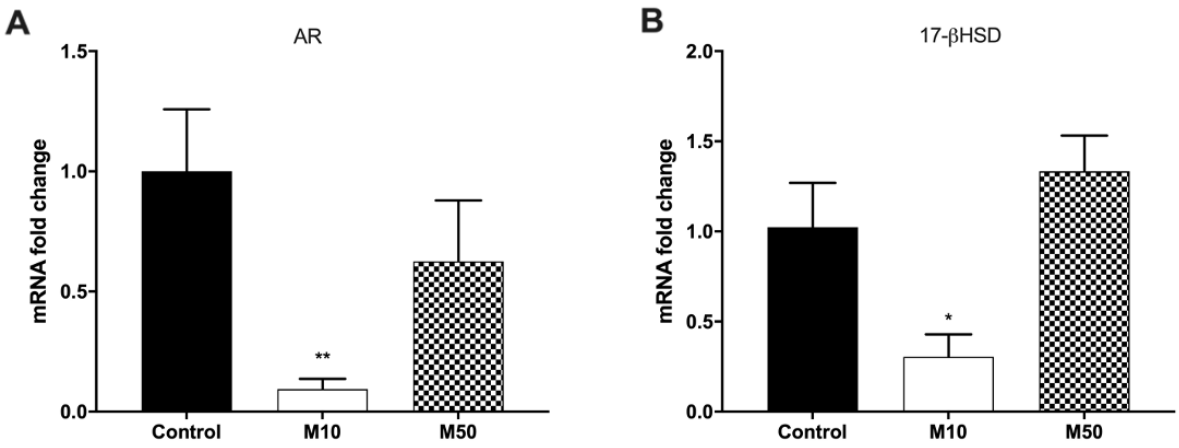

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5 ARTIGO 2

Low malathion concentrations reduce testosterone biosynthesis by Leydig TM3 cells by altering cellular redox profile and inducing oxidative damage

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Low malathion concentrations reduce testosterone biosynthesis by Leydig TM3 cells by altering cellular redox profile and inducing oxidative damage

Rafaela Pires Erthal[1,2], Débora Hipólito Quadreli[1], Tiago Henrique Zaninelli[2,] Waldiceu Aparecido Verri Junior[2], Glaura Scantamburlo Alves Fernandes[1*]

1 – Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

2 – Department of General Pathology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

***Corresponding author**

Rafaela Pires Erthal

Tel.: +55 43 999131971

E-mail address: rafaelaperthal@gmail.com (R. P. Erthal)

Abstract

Malathion is an organophosphate pesticide used in agriculture and control of the *Aedes aegypt* mosquito. Since it has been reported that malathion can impair testosterone production in *in vivo* models, the aim of the present study was to elucidate the damage mechanisms involved in impairing Leydig cell function, given its importance for male reproductive function. To this end, murine Leydig TM3 cells were exposed to concentrations of 1, 10 and 100 μM malathion for 24 hours for evaluation of the compound on cell viability, testosterone biosynthesis, levels of cytokines IL-1β, IL-6, IL-10 and TNF-α, as well as evaluation of the redox profile. Malathion impaired cell viability in a concentration-dependent manner. On the other hand, the lowest malathion concentrations (1 and 10 μM) were the ones that impaired testosterone biosynthesis by TM3 cells. Although the level of IL-1 increased and level of TNF-a decreased at certain concentrations, they were not shown to be related to altered hormone production. We found that different concentrations of malathion induced oxidative stress by increasing superoxide anion and increased antioxidants in a compensatory manner. Finally, we conclude that the altered oxidative profile of TM3 cells caused impairment in their function manifested by reduced testosterone biosynthesis at lower malathion concentrations.

Keywords: malathion, testosterone, oxidative stress, TM3, cytokines

1. Introduction

Infertility is considered a public health problem by the World Health Organization (WHO), affecting approximately 15% of couples of reproductive age (Vayena et al., 2009). Infertility is defined as the inability to achieve conception after 12 months of frequent sexual intercourse (Datta et al., 2016). It is recognized that the male component contributes 50% to such cases of infertility (Mehra et al., 2017) and may be related to dysfunctions of organs such as the pituitary gland and the male gonads. Testicular function includes the production of testosterone by Leydig cells, an androgen necessary for spermatogenesis (Silva et al., 2001).

The etiology of male infertility is 40-50% idiopathic, with the remaining reversible cases being related to anatomical and/or hormonal changes (Agarwal et al., 2021; Cram et al., 2001). In cases where infertility remains with unknown etiology, one should consider unmasking the genetic, environmental, and lifestyle factors capable of interfering with the phenotype of male infertility (Concepción-Zavaleta et al., 2022; Gunes and Esteves, 2021). Several modifiable factors impair male fertility, including air pollution, use of pesticides and harmful chemicals, and exposure to excessive heat (Kumar and Singh, 2022). Thus, the impact of environmental contaminants on male reproductive health must be assessed. Most environmental pollutants damage the male reproductive system by acting as endocrine disruptors or via oxidative stress.

Endocrine disruptors are chemicals capable of interfering with the endocrine system and contributing to male reproductive dysfunction that is highly dependent on androgens (Dohle et al., 2003; Sweeney et al., 2016). Testosterone is the major hormone produced by the testes and is responsible for maintaining spermatogenesis and inhibiting germ cell apoptosis in adulthood (Dohle et al., 2003). Testicular cells express large amounts of androgen receptors (AR) (Zhou et al., 2002) responsive to luteinizing hormone (LH) that stimulate steroidogenesis in Leydig cells (Dankers et al., 2013). Alteration in the function of these cells can impair testicular function and lead to infertility (Yang et al., 2015). To better understand the specific response of Leydig cells after exposure to chemicals, Leydig cells of the TM3 lineage have been used (Gonçalves et al., 2018; Sychrová et al., 2022). TM3 cells are non-tumor cells that possess AR receptors and perform testosterone biosynthesis from cholesterol via CYP11A1 enzyme assembly (Matfier, 1980).

An environmental pollutant noted in a previous study for impairing testosterone synthesis by Leydig cells in rats is malathion (Erthal et al., 2020). Malathion is an organophosphate widely used in food cultivation (EPA, 2006) and its use also recommended by WHO for control of arboviruses vector mosquitoes (WHO, 2016). The Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) recommend that the maximum residue allowed in food crops is 8 mg/L of malathion (equivalent to 24 μM) to avoid possible complications (FAO, 1997).

Previous studies from our group have shown that malathion can impair morphofunctional aspects of both testis (Erthal et al., 2020a) and epididymis (Erthal et al., 2020b) through altered hormone levels, oxidative and inflammatory profiles. It has been pointed out that the altered oxidative profile has great importance in malathion-mediated reproductive damage (Lasram et al., 2014). In vitro study showed that goat testis exposed to malathion for 8 hours (100 ng/mL) impaired the activity of the antioxidant enzymes superoxide dismutase and catalase (Sharma and Alka, 2013).

The compound is also indicated for its genotoxic effects to HepG2, WRL-68, peripheral blood mononuclear cell (PBMC) cell lines and human lymphocytes exposed *in vitro* to malathion (Hernández-Toledano et al., 2020; Olakkaran et al., 2020). Other studies point to malathion for inducing oxidative stress and causing damage to cell lines exposed *in vitro*, such as erythrocytes, human lymphocytes, and oocytes (Durak et al., 2009; Flores et al., 2017; Olakkaran et al., 2020). Although there are studies pointing out the harmful effects of malathion *in vitro* on some cell lines, including the damage of oocytes exposed in vitro to the compound, there are no studies evaluating the damage of in vitro exposure of TM3 cells to malathion and its mechanisms.

Elucidating the mechanisms of damage due to exposure to a particular compound helps in finding interventions and ways to prevent further cellular damage. To better understand the mechanism of malathion's impairment to the male reproductive system, it is important to verify its endocrine disrupting potential, its ability to alter cellular redox state and cytokine levels, as well as to modulate the expression of genes linked to the endocrine function of TM3 cells. Since Leydig cells are essential for maintaining male reproductive function, these data are of great importance since they help elucidate the effects and deleterious mechanisms of malathion on these cells.

2. Materials and methods (Rever se citei 48 hrs também, ou se dei a entender que tinha outra tempo)

2.1. Chemicals

Malathion (dietil-dimetoxitiofosforiltio; CAS no. 121-75-5; Cheminova) was obtained from Dominus Quimica (Jandaia do Sul, Brazil). Malathion was diluted to experimental concentrations with culture medium.

2.2. Cell culture

The mouse (Mus musculus) Leydig cell line TM3 (ATCC® CRL-1714™) was kindly provided by Prof. Dr. Wamberto Antonio Varanda, from the Department of Physiology, Faculdade de Medicina de Ribeirão Preto/USP. This cell line will be cultured in Dulbecco's Modified Eagle Medium: F-12 Ham (DMEM/F12; Gibco ®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine

Serum (SBF, Gibco ®) and 1% penicillin/streptomycin (Gibco ®) and maintained in a humidified incubator containing 5% CO2 at 37°C. Under these conditions, cell viability remains high (>90%).

2.3. Concentrations of malathion

The malathion concentrations evaluated in our study were chosen according to a study by Bonilla et al. (2008) in which they evaluated the *in vitro* malathion exposure in oocytes from mouse (*Mus musculus*). The concentrations chosen were based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and nitroblue tetrazolium (NBT). For the cell viability and superoxide anion production assay, malathion concentrations 1, 10, 100 and 1000 μM were used. Subsequently, 1, 10 and 100 μM malathion concentrations were applied for quantification of testosterone concentration, cytokine level and oxidative profile evaluation.

2.4. The cell viability assay

Cell viability was assessed using the MTT assay. This method is based on the conversion of MTT (Invitrogen, Life Technologies) into formazan crystals by mitochondrial dehydrogenase in living cells (Mosmann, 1983). Cells in exponential growth (1.5×10^5/well in 100 μl of culture medium) were seeded in 96-well culture plates and incubated at 5% CO_2 and 37 ºC for 24 h. After that, various concentrations of malathion were added, and the cells remained in culture for 24 h. Next, the media were removed, and 100 μL of MTT reagent (0.5 mg/mL) was added and incubated for 2 h at 37 ◦C for formazan crystal formation. The cell culture medium was aspirated and 100 μL of DMSO was added to dissolve the crystals. The absorbance was measured at 570 nm in a spectrophotometer (Thermo™ Plate). The experiment was conducted with eight wells per treatment. The percentage of cell viability was calculated according to (Huang et al., 2005).

2.5. Testosterone levels

Cells were seeded in a 96-well plate at a density of 1.5 x 10^5/well. After incubation, malathion was added and remained in culture medium for 24h at the above concentrations. Then the culture medium was collected and frozen at -80°C until the day of analysis. Total testosterone was quantified using commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Plates were read at 415 nm using spectrophotometer (Multiskan GO, Thermo Scientific). Results were expressed in pg/mL.

2.6. Cytokine measurement

Analysis of cytokine levels was performed using supernatant from TM3 cell culture, collected after 24 hours of malathion exposure. IL-1β, IL-6, IL-10 and TNF-α were quantified using commercial ELISA kits (eBioscience, San Diego, CA, USA). Plates were read at 450 nm using a spectrophotometer (Multiskan GO, Thermo Scientific). Results were expressed as picograms (pg) of each cytokine/mL.

2.7. Oxidative profile evaluation of ovaries and uterus

2.7.1. Supernatant preparation

Cells were seeded in a 6-well plate at a density of 1.25 x 10ˆ6/well. After the 24 hours of incubation with the different malathion concentrations, the culture medium was discarded and the cell content was immediately homogenized with a Ultraturrax homogenizer (Marconi) in Phosphate Buffered Saline (pH 7.2). The homogenate was normalized to a protein concentration of 1 mg/ml according to the Bradford method and used to determine the levels of thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST), total and reduced glutathione (GT and GSH) and catalase activity.

2.7.2. Superoxide anion production assay

Samples from TM3 cells grown in the presence of malathion were placed in KCl buffer and processed for assessing superoxide anion production. The samples were mixed with NBT solution (1 mg/mL) and incubated for 60 min at 37 °C. The supernatant was discarded and the precipitated formazam was solubilized by 2M KOH and dimethyl sulfoxide (DMSO). The optical density was measured at 600 nm (Multiskan GO Microplate, Thermo Fisher Scientific, Vantaa, Finland). The final values are shown as NBT reduction (OD 600 nm) (Bussmann et al., 2019).

2.7.3. Lipoperoxidation

The TBARS levels lipid peroxidation as described previously by Federici et al. (2007). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, was determined by the difference between absorbance at 535 and 572 nm (Multiskan GO, Thermo Scientific, Vantaa, Finland). The results are expressed as nanomoles of MDA per milligram of protein.

2.7.4. GT and GSH measurement

Reduced glutathione (GSH) and total glutathione (GT) levels were determined according to the method proposed by Rahman et al. (2007), with some modifications, using 5,5'-dithiobis 20-nitro benzoic acid (DTNB) in the homogenate, as evidenced by the yellow colour formation 26. For determination of total glutathione levels (GT), DTNB, nicotinamide-adenine dinucleotide phosphate (NADPH) and glutathione reductase was used in the homogenate. Both GT and GSH levels were measured at 412 nm (Multiskan GO, Thermo Scientific, Vantaa, Finland), and the results were expressed as micromoles/mg of protein. The levels of GSSG and oxidative stress index (OSI) were calculated as described by (Carrara et al., 2019), taking into account the stoichiometry of the reaction.

2.7.5. Glutathione S-transferase

The activity of glutathione S-transferase was evaluated by spectrophotometry using GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and potassium phosphate buffer. Sample absorbance was measured at 340 nm at 40 sec intervals during 5 min, according to Keen et al. (1976).

2.7.6. Catalase activity assay

Catalase activity was determined based on the principle of peroxide dismutation. The absorbance was measured at 240 nm at 15 sec intervals for 1 min 27.

2.7.7. Evaluation of SOD activity

The evaluation of SOD activity was performed as per the method described by Senthilkumar et al. (2021). Homogenates normalized to 1 mg/mL were used as the enzyme source. A reaction mixture containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazolium (NBT) (96 µM), and Triton X-100 (0.6%) was prepared and incubated for 2 min with 20 mM hydroxylamine hydrochloride (NH2OH· HCl) at pH 6.0. The final volume was adjusted to 200 µL. The reaction consists of quantifying the complex formed between superoxide anions by the addition of NBT and NH2OH· HCl with yellow coloration and the reduction of NBT, forming a blue colour reading at 560 nm. The samples were read every 15 s for 2 min.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) with post hoc Dunnett's test or the non-parametric Kruskal–Wallis test with the Dunn's post hoc test was used to compare the results between the

experimental groups. The Barttlett`s test was performed to evaluate the variance among the experimental groups and the normal distribution was compared using Shapiro-Wilk test. Data are presented as the mean ± s.e.m. Differences were considered significant when $p < 0.05$. The statistical analyses and graph design for the results were performed using GraphPad Prism for Windows (version 7.01 – GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Malathion decreases the viability of TM3 cells and induces superoxide anion generation

Malathion was found to reduce the viability of TM3 cells in a concentration-dependent manner. The MTT assay showed that all concentrations were sufficient to impair this parameter (Figure 1.A). To determine the concentrations, the NBT assay was performed and it was found that the concentrations of 10 and 100 μM increased the generation of superoxide anion by Leydig cells compared to the MEM group, unlike the concentrations of 1 and 1000 μM that showed no change (Figure 1.B).

3.2. Low concentrations of malathion caused greater impairment in testosterone production by TM3 cells

Figure 2 shows the testosterone concentrations after exposure to different concentrations of malathion. Although the highest concentration tested (100 μM) did not alter testosterone production by Leydig cells, the lower concentrations (1 and 10 μM) significantly decreased androgen concentrations compared to the control group (MEM).

3.3. Different malathion concentrations impair pro-inflammatory cytokine levels

Figure 3 shows that none of the malathion concentrations used impaired IL-6 and IL-10 levels compared to the MEM group. On the other hand, malathion 100 uM significantly increased IL-1β levels and malathion 1 μM decreased TNF-α levels relative to the control group. The concentrations did not favor changes in the levels of these pro-inflammatory cytokines.

3.4. Malathion compromises do oxidative profile in TM3 cells exposed for 24 hours

The oxidative profile of TM3 cells exposed do different concentrations of malathion is presented in Figure 4. Although only the malathion (100 μM) significantly increased MDA levels compared to MEM, the percentage of oxidative stress index was increased by both malathion 10 and 100 μM in Leydig cells line. Regarding the glutathione system, GSH levels were increased by exposure to malathion 10 μM while GT and GSSG were increased by both malathion 10 and 100 μM concentration relative to control. The GST activity was also increased by malathion 100 μM, but not by the other

concentrations. The catalase activity was also increased by malathion 1 or 10 μM, but not by malathion 100 μM compared to MEM.

4. Discussion

The present study showed that concentrations of malathion tolerated by the EPA as a residue in food were sufficient to impair Leydig cell viability and compromise testosterone levels at lower concentrations. This impairment of TM3 cell line function occurred concomitantly with altered pro-inflammatory cytokine profiles in a malathion dose-dependent manner, as well as altered cellular redox state.

The cell viability of TM3 cells proved more susceptible to the action of malathion compared to the peripheral blood mononuclear cell line (PBMCs). Study showed that such cells exposed to malathion concentrations up to 100 μM for 48 hours did not impair cell viability (Hernández-Toledano et al., 2020). On the other hand, the same study pointed out that concentrations starting at 1 μM of malathion were already sufficient to impair the viability of liver cells of the HepG2 and WRL-68 cell lines, confirming that there are cell lines that are more sensitive to the action of the organophosphate.

The resulting impairment of Leydig cell viability observed in the present study was reflected in the reduction of their functionality observed through reduced testosterone levels. Leydig's cells are between the seminiferous tubules in testicles. They internalize cholesterol into the mitochondria, where steroidogenesis will occur. This event happens due to the enzymatic apparatus expressed by the Leydig cells, involving CYP11A1, 3βHSD2, CYP17A1 and the enzyme 17βHSD3, which will do the final conversion into testosterone (Zirkin and Chen, 2000). In addition to producing androgens, Leydig cells in differentiation possess androgen receptor (AR), being sensitive to the action of testosterone, mainly enabling the development and differentiation of these cells during the puberty of the individual (Shan et al., 1997). Testosterone, in turn, also acts on ARs of Sertoli cells and spermatogonia, providing support for spermatogenesis and maintenance of male fertility (Walker, 2011).

Interestingly, the lower concentrations of malathion (1 and 10 μM) were the ones that reduced the levels of the hormone, but not the 100 μM concentration. Some endocrine disruptors, such as Bisphenol S have also been noted to cause greater impairment of rat testicular function when in lower doses through conformational alteration of proteins involved in the steroid hormone cascade (Darghouthi et al., 2022). The same effect was observed with the compound dichlorodiphenyltrichloroethane (DDT), capable of causing greater damage to steroidogenesis when exposed to low doses (Yaglova et al., 2021). However, there are no studies to date that showed this same effect after exposure to malathion.

The cytokine imbalance observed in the present study can also be correlated with altered testosterone levels. The altered cytokines in the present study, IL-1β and TNF-α, although normally

categorized as markers of inflammation, play physiological roles in Leydig cells through modulation of the hypothalamic-pituitary-gonadal axis (Bornstein et al., 2004; Hales et al., 1999; Svechnikov et al., 2001). However, this modulation is excluded in the present study given the *in vitro* experimental model used.

In addition, increased concentrations of TNF-α may be directly related to decreased cell viability, enabling down-regulation of steroidogenic enzyme production as a response to damage (Leisegang and Henkel, 2018; Wu et al., 2012). In the present study, the decrease in TNF-α levels observed at the lowest experimental concentration of malathion was not associated with decreased testosterone levels, showing that other mechanisms were involved in this disorder.

IL-1β has also been pointed out to impair the viability of TM3 cells (Leisegang and Henkel, 2018). On the other hand, the influence of this interleukin on testosterone synthesis is quite controversial. While there are studies showing that IL-1β reduces testosterone synthesis by Leydig cells (Calkins et al., 1988; Lin et al., 1991), other studies show that IL-1β did not alter (Sun et al., 1993) or even increased testosterone synthesis (Verhoeven et al., 1988). In our study, the increased level of IL-1β was not related to altered hormone levels. Thus, we conclude that altered cytokine levels were not the mechanism involved in the decreased testosterone levels in this experimental model. Nevertheless, an in vitro study showed that increased MDA levels were associated with increased levels of the cytokine IL-1β in sperm cells (Martínez et al., 2007). The same correlation was made in the present study, since increased MDA and IL-1b levels were only observed at the highest concentration of malathion (100 μM) in TM3 cells.

In the present study, we showed that concentrations of 10 and 100 μM malathion were sufficient to increase the production of superoxide anion and consequently the index of oxidative stress. The altered redox state can result from the imbalance between prooxidants and antioxidants. The accumulation of oxidative damage in intracellular macromolecules then contributes to cellular functional deficit (Finkel and Holbrook, 2000). Leydig cells produce reactive oxygen species (ROS) from several sources, including the mitochondrial electron transport chain and enzymatic reactions involving cytochrome P450 (Hanukoglu, 2006). On the other hand, the same authors point out that the increase of scavenger molecules and antioxidant enzyme activity have protective effects on steroidogenic cells. Interestingly in the present study, levels of GSH, GSSG and GST enzyme activity were increased in TM3 cells exposed to 10 and 100 μM malathion as a response to increased oxidative stress, seeking redox balance.

An *in vivo* study demonstrating the effects of exposure of male rats to malathion (100, 500 and 1500 ppm for 4 weeks) showed that as the malathion doses increased, so did the antioxidant power in plasma, but in saliva there was no change (Abdollahi et al., 2004). This result showed that the antioxidant reaction to malathion was locally dependent. Another *in vivo* model involving exposure of *Cyprinus carpio* to malathion (0.5 and 1 mg/L) for 14 days also showed increased activity of the enzymes SOD, catalase and GST in liver and kidney (Mişe Yonar et al., 2017). These studies corroborate the present

study and make it possible to state that there was an alteration in the entire redox profile especially of the cells exposed to concentrations of 10 and 100 μM of malathion. We also emphasize that there were no studies that presented the redox profile of Leydig cells exposed to malathion.

Studies have shown the negative effects of oxidative stress and reduced GSH levels on androgen production by both adrenal (Abidi et al., 2008) and TM3 cells (Chen et al., 2015) through inhibition of the MAPK pathway. The results presented herein first indicate that the increased activity of the GST enzyme and of the GSH and GSSG molecules point to their protective role in maintaining normal testosterone biosynthesis in TM3 cells exposed to malathion 100 μM. In contrast, the lower action of antioxidant systems at the lower malathion concentration (1 μM) favors a pro-oxidant environment and impairs testosterone production.

We observed that the lower concentrations of malathion were more detrimental to Leydig cell function. This approach would represent a paradigm and new models of exposures to low doses of environmental chemicals need to be evaluated. In addition, the expression of genes involved in testosterone metabolism and biosynthesis need to be checked to better understand the mechanism of injury involving oxidative stress and testosterone production.

5. Conclusion

We conclude from the data presented that exposure of TM3 cells to low concentrations of malathion was most important in reducing testosterone biosynthesis through impairment of the redox state. We further point out that malathion is present in the environment and the concentrations used in the present study were based on the maximum allowable residual limit being of importance in mimicking human exposure.

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Figure legends

Figure 1. Determination of malathion concentration to be used in TM3 cell lineage for 24 hours. (A) Percentage of cell viability (%) of Leydig cells (TM3) exposed to malathion 1, 10 or 100 μM for 24 hours as measured by MTT assay. (B) Generation of superoxide anion by Leydig (TM3) cells exposed to increasing concentrations of malathion for 24 hours measured by NBT assay.
Results are presented as mean ± S.E.M. ANOVA test with *a post hoc* Dunnett's test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. MEM: minimum essential medium as a negative control; Triton: positive control.

Figure 2. Testosterone production by Leydig cells (TM3) exposed to malathion (1,10 and 100 μM) for 24 hours.
Results are presented as mean ± S.E.M. ANOVA test with *a post hoc* Dunnett's test. *p<0.05. MEM: minimum essential medium as a negative control.

Figure 3. Cytokine levels in Leydig cells (TM3) exposed to malathion (1, 10, and 100 μM) for 24 hours. (A) IL-1β, (B) IL-6, (C) TNF-α and (D) IL-10 production determined by ELISA assay using TM3 cells supernatant.
Results are presented as mean ± S.E.M. ANOVA test with *a post hoc* Dunnett's test. *p<0.05. MEM: minimum essential medium as a negative control.

Figure 4. Redox profile of Leydig cells (TM3) exposed to malathion (1, 10 and 100 μM) for 24 hours. (A) MDA levels, (B) Oxidative stress index (%), (C) GSH, (D) GT and (E) GSSG levels, (F) GST and (G) Catalase activity
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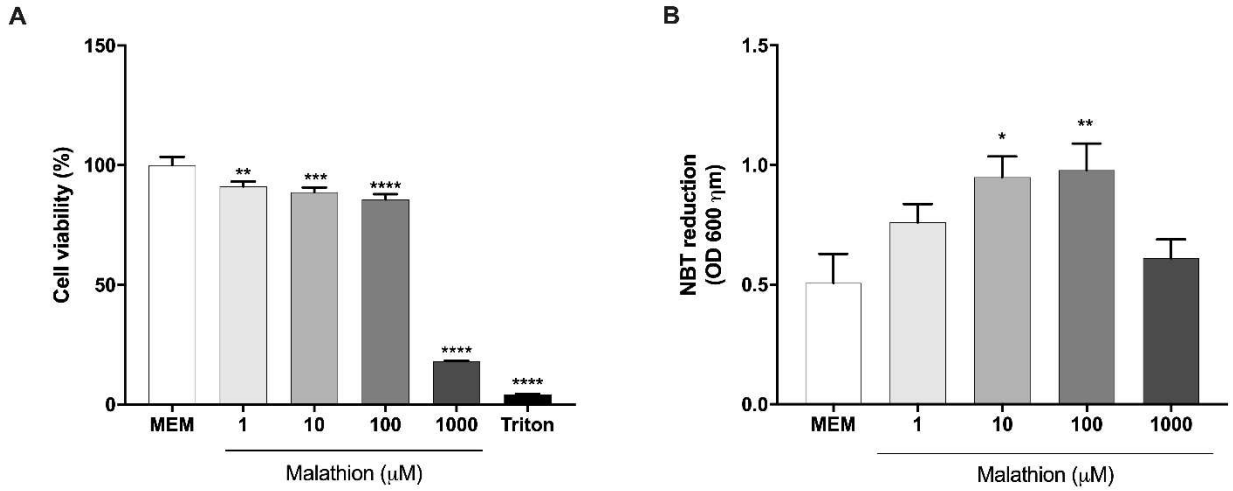

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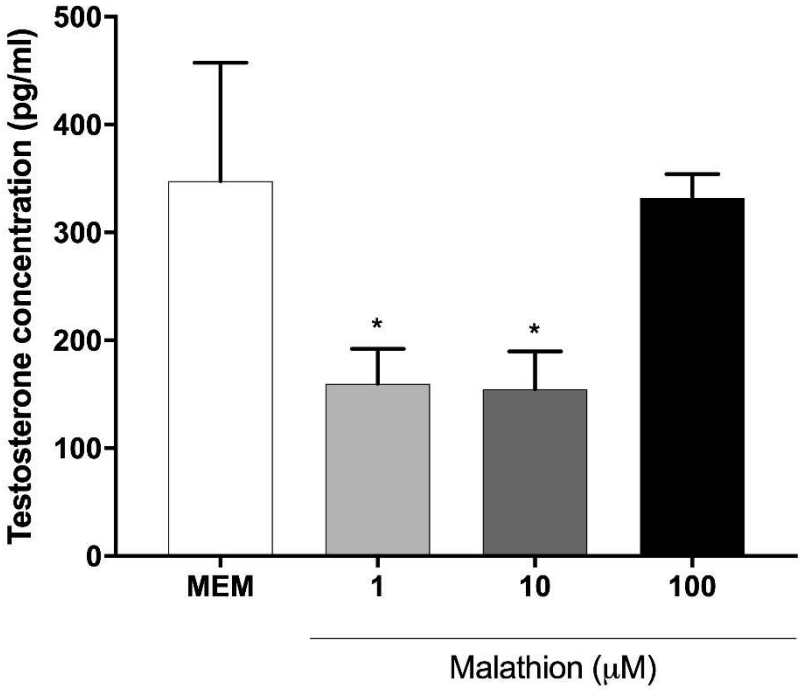

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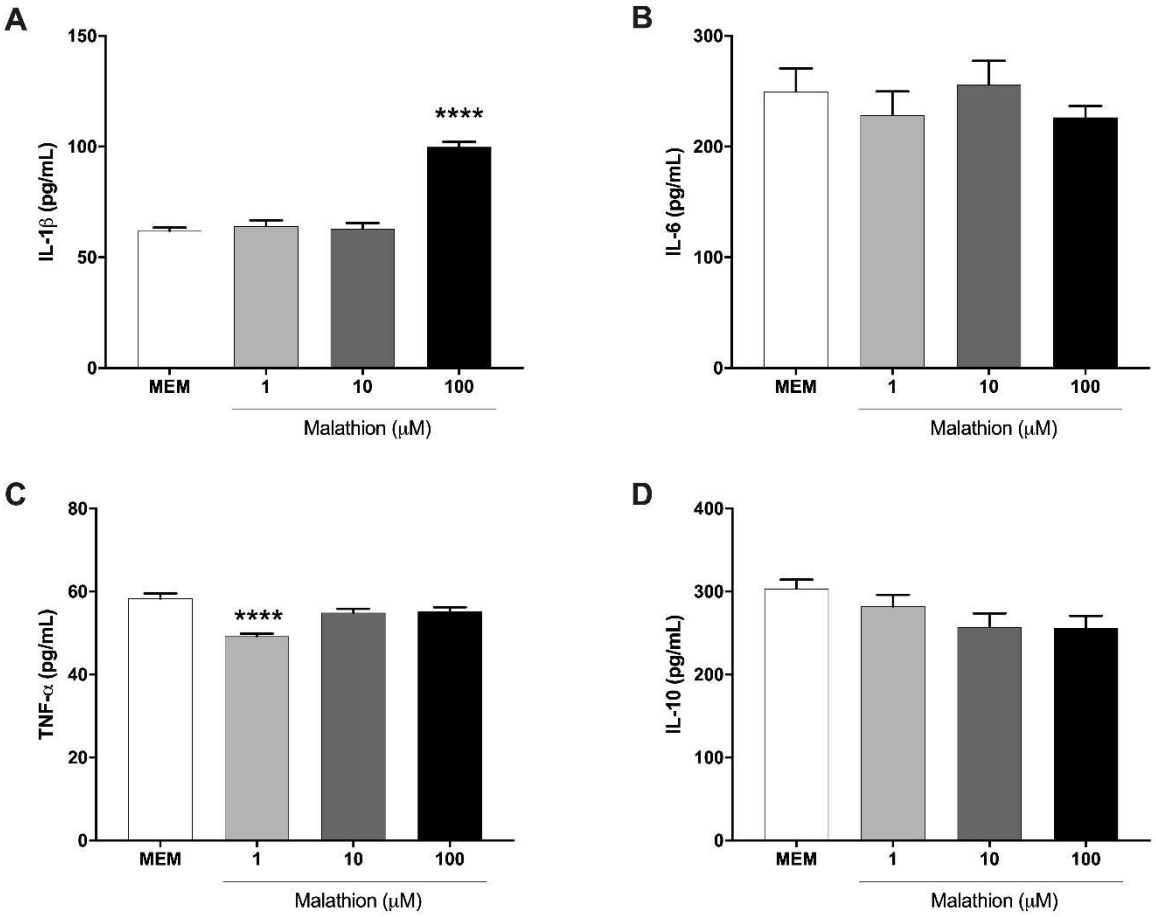

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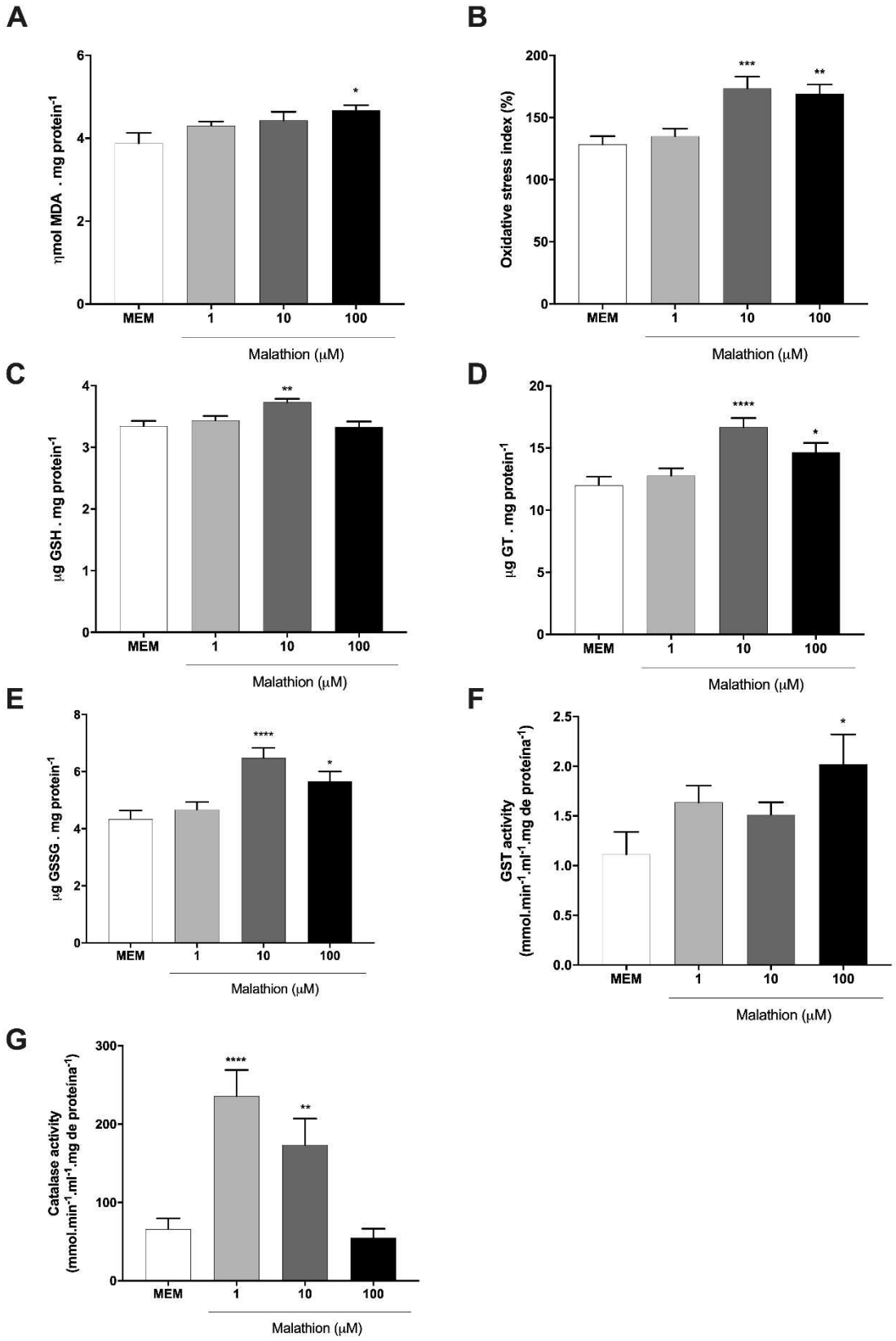

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6 ARTIGO 3

Malathion impairs folliculogenesis and follicle ovarian integrity by reducing estradiol of in vitro cultured bovine preantral follicles

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Malathion impairs folliculogenesis and follicle ovarian integrity by reducing estradiol of *in vitro* cultured bovine preantral follicles

Rafaela Pires Erthal[1,2], Larissa Zamparone Bergamo[3], Giovanna Fachetti Frigoli[1], Dayane Priscila dos Santos[1,2], Andrea Name Colado Simão[4], Marcelo Marcondes Seneda[3], Glaura Scantamburlo Alves Fernandes[1*]

1 – Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

2 – Department of General Pathology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

3 – Department of Veterinary Clinics, Agrarian Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970

4 – Department of Pathology, Clinical Analysis and Toxicology, Health Sciences Center, University Hospital, State University of Londrina – UEL, Londrina, Paraná, Brazil, Av Robert Koch, 60, Postal code: 86038-440

Running head: Ovarian explant damaged by low doses of malathion

***Corresponding author**

Glaura Scantamburlo Alves Fernandes

Tel.: +55 43 33714417

E-mail address: glaura@uel.br (G.S. A. Fernandes)

Abstract

Malathion is an organophosphate insecticide used in agriculture for controlling vector-borne diseases, such as dengue and Zika virus infection. Humans and animals, especially those living in rural environments, are exposed to such compounds through the ingestion of contaminated food, inhalation of contaminated air, or skin contact. The aim of the present study was to evaluate the direct effect of malathion on pre-antral follicles in an *in vitro* model. Ovaries from Nelore cows (n = 5) were divided into fragments (n = 11 per ovary) and cultured for 44 h in a minimal essential medium (MEM+) or with 500 or 1000 μM of malathion (M500 or M1000). One fragment per group was fixed in Methacarn and processed for histological analysis. Other fragments were used to evaluate the oxidative profiles. After 44 h, the culture medium was collected and used for the evaluation of estradiol levels. Although malathion concentrations did not alter the oxidative profile, they decreased the estradiol levels and compromised the pre-antral follicular morphometry and integrity. Furthermore, the M1000 group exhibited impaired follicular development. From the present work, we can conclude that exposure to doses of malathion incapable of generating oxidative stress is enough to harm the integrity and growth of preantral ovarian follicles in bovines in an in vitro model. Unlike the results of previous studies, our study suggests that these changes occur via the reduction of estradiol.

Keywords: malathion, ovarian follicle, endocrine disruptor, bovine

1. Introduction

The in vitro culture of preantral follicles has increased the possibility of evaluating fertility parameters, as it allows the investigation of factors related to follicular development (Max et al., 2018). This method mimics the physiological events in vivo by presenting a specific medium supplemented with substances that enable the activation, development, and maintenance of follicular integrity and adjacent ovarian stromal cells (Silva et al., 2016). In mammals, preantral follicles represent approximately 90% of the entire population of ovarian follicles, although the vast majority do not reach the ovulation stage, reaching the stage of follicular atresia (Guilbault et al., 1986). This *in vitro* tissue culture model is an important tool for the study of reproductive toxicology as it allows the assessment of damage not only of isolated cell cultures, but specifically on a tissue fragment and suggests mechanisms by which compounds can interfere with fertility (Brevini et al., 2005).

Previous studies from our lab have shown that malathion impairs testicular (Erthal et al., 2020a) and epididymal (Erthal et al., 2020b) morphophysiological aspects through hormonal alteration and oxidative profiling of male rats exposed during puberty. Regarding the female reproductive system, it is clear from the literature that compounds from the organophosphate class can compromise its morphophysiology. Malathion is an organophosphate with a relatively large spectrum of activities across a wide range of agricultural food and feed crops (EPA, 2006). The World Health Organization (WHO) has recommended the use of malathion to control insect vectors of diseases, such as malaria, dengue, Zika, and chikungunya (WHO, 2016). Although the neurogenic mechanism of action of this compound occurs through the inhibition of the enzyme acetylcholinestasis, some studies point to the generation of oxidative stress as a mechanism of tissue damage (Brocardo et al., 2005; Fortunato et al., 2006).

An in vivo study showed that low doses of malathion (33 mg kg-1) decreased the number of healthy follicles and increased the number of atretic follicles in rats exposed for 15 days (Koc et al., 2009). Arab et al. (2018) reported malathion as an inducer of reactive oxygen species in the ovaries of adult rats exposed to a dose of 50 mg kg-1 for two weeks (Abbasabad Arab et al., 2018).

A study in an in vitro model of porcine granulosa cells exposed to malathion showed that the compound induced apoptosis via oxidative stress in a dose-dependent manner (25–175 μM) (Wang et al., 2018). Another in vitro model using even lower concentrations of malathion (100 μM) in granulosa cells from goats also showed induction of apoptosis as a consequence of oxidative stress (Bhardwaj and Saraf, 2014). Interestingly, the lethal concentration of malathion 50 was 1mM in oocytes cultivated in vitro, and the compound was effective in impairing in vitro fertilization and embryo development (Ducolomb et al., 2009).

Due to the lack of studies addressing all aspects involving follicular response to malation and the great importance of this compound for human exposure, the present study aimed to evaluate whether ovarian follicles from Bos taurus can impair estradiol biosynthesis, as well as follicular morphology and its oxidative profile

2. Materials and Methods

2.1 Collection and transport of ovaries

Ovaries (n = 10) were collected from five adult *Bos indicus* females from a local slaughterhouse that were cyclical, judging by the presence of the corpus luteum, and in body condition (body score of 2.5–3.5 on a scale of 0–5). After recovery, the ovaries were washed in 70% ethanol and phosphate-buffered saline (PBS) solution, processed, and transported to a temperature-controlled laboratory (20–24°C; approximately 15 km). Bovine ovaries were transported using minimal essential medium (MEM, osmolarity 300 mOsm/L, pH 7.2; Gibco BRL, Rockville, MD, USA) supplemented with 200 mg/mL penicillin and 200 mg/mL streptomycin.

2.2 Cultivation of preantral follicles and experimental protocol

The ovaries from each animal were carefully processed, the surrounding tissues and ligaments were removed, and then the ovary was sectioned longitudinally. The medulla, large antral follicles, and corpora lutea were removed. Subsequently, the cortex of the ovary was divided into fragments of

approximately 3 × 3 × 1 mm according to the method proposed by Bizarro-Silva et al. (2018), and those with visible corpora lutea or large antral follicles were discarded (Figure 1).

Fragments of the ovarian cortex were cultured individually in 1 mL aliquots of culture medium in 24-well culture plates in an incubator at 38.5 °C in an atmosphere of 5% carbon dioxide (CO_2) in air and saturated humidity. The control culture comprised of MEM (osmolarity 300 mOsm/L, pH 7.2; Gibco BRL, Rockville, MD, USA) supplemented (MEM+) with insulin–transferrin–selenium (ITS) (6.25 mg/mL insulin, 6.25 mg/mL transferrin, and 6.25 ng/mL selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA; Gibco BRL, Rockville, MD, USA), 20 UI/mL penicillin, and 200 mg/mL streptomycin.

For malathion exposure, MEM+ was supplemented with the compound at concentrations of 500 and 1000 μM (M500 and M1000, respectively). The cultivation period and malathion concentrations used were determined based on previous studies of follicle exposure to pesticides (Casas et al., 2010; Flores et al., 2017). Malathion (dietil-dimetoxitiofosforiltio; CAS no. 121-75-5; Cheminova) was obtained from Dominus Quimica (Jandaia do Sul, Brazil). Ten fragments of the ovarian cortex of each animal were cultured in the medium and tested after 44 h.

2.3 Histological processing

For the analysis of ovarian morphology and ovarian follicles, ovarian cortex fragments were cultured for 44 h and fixed by immersion in Methacarn solution (10% acetic acid, 60% methanol, and 30% chloroform; Sigma-Aldrich Co., St. Louis, Missouri, USA) for 4 h. After fixation, the tissues were kept in 70% ethanol for 24 h and dehydrated in a graded series of ethanol, clarified, cleared in xylene, and embedded in Paraplast® (Sigma-Aldrich Co., St. Louis, Missouri, USA) for preparation of blocks for histological analysis. Subsequently, each block was sectioned to a thickness of 5 μm with an interval of five sections of tissues with a rotary microtome (Leica®, Wetzlar, Germany) to mount on microscope slides. The slides were stained with hematoxylin and eosin and examined using light microscopy.

2.4 Histopathological analysis and assessment of in vitro folicular growth

All sections were examined under a light microscope (Motic®, Carlsbad, California, USA) at 40x and 100x magnification and were performed by the same person throughout the study. Follicles were ranked according to the following detailed specifications: Preantral follicles were classified as: (1) primordial (one layer containing somatic cells, known as granulosa cells, flat or flattened around the oocyte) or in development; (2) primary (a single layer of cuboidal granulosa cells around the oocyte), or (3) secondary (two or more layers of cubic granulosa cells). To evaluate the activation and growth of follicles, they were quantified at different stages of development (primordial, primary, and secondary) in control and after in vitro cultivation in different treatments.

Follicles were also classified according to their morphology integrity based on their structure, which was either intact or degenerated. The follicle was considered to be morphologically intact when it had a regular nucleus and was surrounded by granulosa cells arranged in discrete layers, while it was called degenerated if it was huddled with a pyknotic nucleus and surrounded by granulosa cells isolated from the disorganized basement membrane (Búfalo et al., 2016). Approximately 250 follicles per treatment were studied for the duration of cultivation. To prevent recounts, preantral follicles were counted only in the section in which the nucleus of the oocyte was observed. To evaluate the growth rates and follicular activation, only intact follicles were considered, and the percentages of primordial, primary, and secondary follicles were calculated after 44 h of culture at varying concentrations of malathion in the culture medium.

2.5 Morphometry analysis of preantral follicles cultured in vitro

For the analysis of follicles and cultured oocyte diameters, 30 morphologically intact preantral follicles were observed per treatment (MEM+, M500, and M1000) among the five replicates, and images were captured. The measurements of follicles and oocytes were performed according to the method proposed by Silva-Buttkus et al. (2008), and the oocyte and follicular diameters were calculated from the arithmetic mean of two perpendicular measurements using the BELview software (v.6.2.3.0 for Windows).

2.6 Estradiol analysis

To evaluate follicular steroidogenesis in vitro, estradiol concentrations were measured in spent culture media after 44 h. The total estradiol present in the media was measured using chemiluminescence (Architect System, Abbott, Wiesbaden, Germany), according to the manufacturer's recommendations. The intra-assay coefficient of variation and minimum sensitivity of the assay were 4.6 % and 0.15 nmol/L, respectively.

2.7 Evaluation of oxidative profile

2.7.1 Tissue preparation

The frozen fragments were homogenized in 50 mM Tris-hydrogen chloride (HCl) buffer (pH 7.4) and normalized to 1 mg/mL protein concentration according to the Bradford method (Bradford, 1976). The homogenate was used to determine the levels of thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST), reduced glutathione (GSH), catalase, and superoxide dismutase (SOD).

2.7.2 TBARS assay

TBARS levels lipid peroxidation as previously described (Federici et al., 2007). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, were determined by the difference between the absorbance at 535 and 572 nm (Multiskan GO; Thermo Scientific, Vantaa, Finland). The results were expressed as nanomoles of MDA per milligram of protein.

2.7.3 GST activity

The activity of glutathione S-transferase was evaluated spectrophotometrically using GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and potassium phosphate buffer. Sample absorbance was measured at 340 nm at 40 sec intervals for 5 min, according to Keen et al. (1976).

2.7.4 GSH measurement

GSH levels were determined according to the method proposed by Rahman et al. (2006), with some modifications, using 5,5'-dithiobis 20-nitro benzoic acid in the homogenate, as evidenced by the yellow color formation (Rahman et al., 2007). GSH levels were measured at 412 nm, and the results were expressed as micromoles/mg of protein.

2.7.5 Catalase activity assay

Catalase activity was determined based on the principle of peroxide dismutation. The absorbance was measured at 240 nm at 15 sec intervals for 1 min (Aebi, 1984).

2.7.6 Evaluation of SOD activity

The evaluation of SOD activity was performed as per the method described by Senthilkumar et al. (2021). Homogenates normalized to 1 mg/mL were used as the enzyme source. A reaction mixture containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazolium (NBT) (96 μM), and Triton X-100 (0.6%) was prepared and incubated for 2 min with 20 mM hydroxylamine hydrochloride ($NH_2OH\cdot HCl$) at pH 6.0. The final volume was adjusted to 200 μL. The reaction consists of quantifying the complex formed between superoxide anions by the addition of NBT and $NH_2OH\cdot HCl$ with yellow coloration and the reduction of NBT, forming a blue color reading at 560 nm. The samples were read every 15 s for 2 min.

2.8 Statistical analysis

The data were initially subjected to normality tests (Shapiro–Wilk) and homogeneity of variance (Bartlett) tests. The results were then subjected to analysis of variance (ANOVA) and the *post hoc* Dunnett's test ($P \leq 0.05$). All analyses were performed using the GraphPad Prism software (Campinas, SP, Brazil), and the values were considered to be statistically significant when $P \leq 0.05$. Statistical analyses and graph design for the results were performed using the GraphPad Prism v.9.01 software for Mac (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean + standard error of the mean (SEM).

3. Results

3.1 Malathion compromises the morphology and growth of ovarian follicles after 44 h of exposure

The exposure of ovarian follicles to malathion at 1000 μM for 44 h altered follicular growth, as presented in Table 1. Although the percentage of primordial and primary follicles did not change after exposure to malathion, both doses of the compound significantly reduced the mean percentage of secondary ovarian follicles when compared to the MEM+ group.

In relation to histopathological analysis, both malathion exposure increased the number of abnormal follicles in relation to the MEM+ group (Table 1). The major abnormalities observed are shown in Figure 2.

In addition, ovarian follicles only in the M1000 group showed a reduction in oocyte diameter compared to the MEM+ group. In contrast, follicular size was not affected by exposure to any concentration of malathion (Figure 3).

3.2 Malathion reduces the estradiol concentration of ovarian follicles after 44 h

The estradiol concentrations measured from the culture median exposed to both malathion concentrations decreased in relation to the MEM+ group (Figure 4).

3.3 Alterations in ovarian follicles are independent of oxidative stress

The oxidative profiles are shown in Table 2. Different malathion concentrations were not sufficient to induce lipid peroxidation levels, as evidenced by TBARS analysis. Furthermore, the levels of GSH and glutathione-S-transferase, catalase, and SOD activity were maintained even after exposure of ovarian follicles to both doses of malathion compared to MEM- or MEM+.

4. Discussion

The present study shows that malathion acts as an endocrine disruptor (ED) at both doses, which may be the mechanism by which it changes the morphophysiological parameters of ovarian follicles in an *in vitro* model. This is an important finding as some articles on EDs do not include malathion or even its respective class as a point of discussion (Schug et al., 2016; Monneret, 2017). Therefore, it is necessary to draw attention to this important environmental pollutant.

Endocrine disruptors are chemical compounds present in the environment that are capable of interfering with the endocrine system of humans and animals, acting directly on the endocrine organs, changing their functions and metabolism or interacting with their hormone receptors (EPA, 1997). However, whether they can cause toxicity at low doses and whether environmental doses pose any risk to human health remain unclear. Such compounds, among the many ways of acting to destabilize the endocrine system, can perform their effects by also acting as estrogen receptor antagonists or by compromising the biosynthesis of steroid hormones in ovarian follicles (Gray et al., 1997). It is not surprising that in the present study, when looking for the mechanism by which malathion can compromise the integrity of ovarian follicles, we found that it acted as an ED. Kiyama and Wada-Kiyama (2015) classified malathion as a pesticide with estrogenic activity capable of impairing the estrogen receptor signaling pathway by inducing estrogen receptor (ER) transactivity and aromatase enzyme activity.

It should be noted that, unlike males, females have fixed numbers of germ cells and, therefore, changes in hormonal levels or activities can definitively compromise folliculogenesis (Marques-Pinto and Carvalho, 2013). In the present study, we observed that the in vitro action of malathion on preantral follicles induced a delay in folliculogenesis due to a decrease in secondary follicles. This result corroborates the study by Casas et al. (2010) who reported a malathion with meiosis resumption blocking effect in the maturation stages of oocytes exposed in vitro for 44 h (100 μM). Furthermore, a study using LC50 and IMC50 (1000 and 750 μM, respectively) for malathion in an in vitro model also showed impairment in the maturation of porcine cumulus-oocyte complexes (Flores et al., 2017).

It is important to emphasize that the toxic effect of malathion on the maturation of preantral follicles observed in the present study may induce the destruction of oocytes and follicular cells, and thus, it can contribute to the reduction of the animal's fertility. In addition to compromising the development of preantral follicles in vitro and causing an impairment of fertility, malathion also harms the follicular morphology by reducing the sizes of the follicles and oocytes and altering their integrity.

The integrity of granulosa and theca cells is crucial for steroidogenesis and oocyte development, and these cells act as targets of EDs (Craig et al., 2011). Since oocyte maturation is a prerequisite for fertilization and development (Pocar et al., 2003), an impairment of this process, as presented in this study, can potentially harm the female reproduction process. Likewise, the involvement of follicular cells observed in the present study may indirectly affect the production of steroid hormones and interfere with the maturation of oocytes (Beker-Van Woudenberg et al., 2004). Another important point to consider is that low doses of other EDs can impair the fertility of men and women from the perspective of endocrinology, which corresponds to the results presented in this study (Vandenberg, 2014; Barouki, 2017). It is also worth noting that low doses mimic the real doses at which humans and animals are exposed to environmental contamination, except workers who deal with the compost daily and are exposed to greater doses.

Unlike previous studies that point to the oxidative stress-inducing role of malathion (Bhardwaj and Saraf, 2014; Wang et al., 2018), the concentrations used in this experimental model were not sufficient to modify the oxidative profile as the biomarkers for oxidative stress and antioxidants did not change

after exposure to different concentrations of malathion. Therefore, the damage caused by exposure to malathion does not occur because of the increased oxidative stress. However, it seems reasonable to imply that the alterations observed in this study are related to the reduction in estrogen levels that reflect the impairment of both the development and integrity of the follicles, which are essential to maintain the quality of female gametes to be disposed to fertilization.

Our findings show that the concentrations used in this model, although insufficient to generate changes in the oxidative profile, compromised hormone levels, resulting in impaired follicular morphology and intergity. Based on these considerations and the damage identified in the ovarian follicle, which is essential for female reproduction, new measures need to be taken seeking new alternatives to the use of malathion

5. Conclusion

From the present work, we conclude that even exposure to doses of malathion that are considered to be incapable of generating oxidative stress was enough to harm the integrity and growth of preantral ovarian follicles in bovines in an in vitro model. Unlike what has been presented so far, these changes occur via the reduction of estradiol.

Conflict of interest

The authors declare that they have are no conflicts of interest.

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Table legends

Table 1. Development and morphological integrity of preantral ovarian follicles from *Bos indicus* impaired after exposure to malathion for 44 hours.

Values expressed as mean ± S.E.M. ANOVA test with the *post hoc* Dunnet's test. * $p<0.05$; **** $p<0.0001$; MEM+, cultivated follicles in median; M500, malathion 500 mg/mL; M1000, malathion 1000 mg/mL.

Table 2. Oxidative profile of preantral ovarian follicles from *Bos indicus* exposed to malathion for 44 hours.

Values expressed as mean ± S.E.M. ANOVA test ($p > 0.05$). MEM+, cultivated follicles in median; M500, malathion 500 mg/mL; M1000, malathion 1000 mg/mL. TBARS, Thiobarbituric acid reactive substance; MDA, Malondialdehyde; GST, Glutathione-S-Transferase; GSH, Reduced glutathione; CAT, Catalase; SOD, superoxide dismutase.

Figure legends

Figure 1. Experimental design
Nine fragments (3mm x 3mm x 3mm) of ovarian follicles per animal (n = 5) were destined to cultivated in median (MEM+) or supplemented with malathion (500 or 1000 mg/mL) groups for 44 hours. All the fragments were destined to histological processing and evaluation of oxidative profile. Estradiol concentration was determined from median of cultured fragments (MEM+, M500 and M1000).

Figure 2. Histopathological analysis of preantral ovarian follicles from *Bos indicus* exposed to malathion for 44 hours.
Photomicrographs of ovarian follicles from MEM+ (A,B), M500 (C,D) and M1000 (E,F) groups. (A and B) The normal aspect of ovarian follicles. (C and E) Acidophilic cells and disorganized granulosa cells (GC) (arrow). (D) Disorganized GC (arrow) and oocyte cytoplasmic retraction (arrowhead). (F) Presence of cytoplasmic vacuole (asterisk). MEM+, cultivated follicles in median; M500, malathion 500 mg/mL; M1000, malathion 1000 mg/mL. *St*, stroma; *GC*, granulosa cells; N, nucleous. Haematoxylin and eosin stains, 400 X magnification.

Figure 3. Morphometry of preantral ovarian follicles from *Bos indicus* exposed to malathion for 44 hours. (A) Follicular size (μm) and (B) Oocyte diameter (μm).

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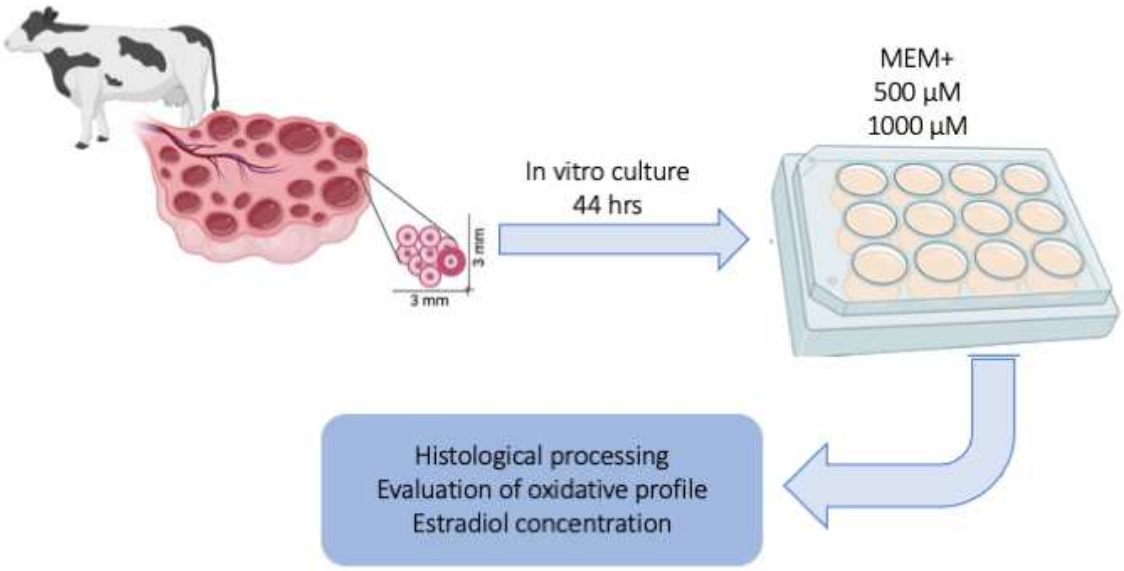
	MEM+	M500	M1000
Stage of development (%)			
Primordial follicles	75.2 ± 3.0	79.4 ± 4.0	84.9 ± 2.5
Primary follicles	16.2 ± 2.4	16.1 ± 3.6	10.5 ± 2.3
Secundary follicles	8.6 ± 1.5	4.5 ± 0.7*	4.6 ± 0.7*
Morphological integrity (%)			
Normal follicles	87.6 ± 1.3	71.2 ± 2.1****	71.6 ± 1.7****
Abnormal follicles	12.4 ± 1.3	28.8 ± 2.1****	28.4 ± 1.7****

Values expressed as mean ± S.E.M. ANOVA test with the *post hoc* Dunnet's test. * $p<0.05$; **** $p<0.0001$; MEM+, cultivated follicles in median; M500, malathion 500 mg/mL; M1000, malathion 1000 mg/mL.

Table 2. Oxidative profile of preantral ovarian follicles from *Bos indicus* exposed to malathion for 44 hours.

Biomarker	MEM+	M500	M1000
TBARS (ηmol MDA.mg protein^{-1})	2.18 ± 0.2	2.23 ± 0.2	2.51 ± 0.1
GSH (µmol.mg protein^{-1})	6.41 ± 0.1	6.31 ± 0.3	6.14 ± 0.2
GST (mmol.min^{-1}.mg protein^{-1})	23.76 ± 8.1	29.44 ± 7.2	16.38 ± 1.0
CAT (mmol.min^{-1}.mg protein^{-1})	97.16 ± 24.0	65.40 ± 20.4	59.79 ± 11.7
SOD (ηmol.min^{-1}.mg protein^{-1})	5.51 ± 0.5	5.58 ± 0.5	6.83 ± 0.2

Values expressed as mean ± S.E.M. ANOVA test ($p > 0.05$). MEM+, cultivated follicles in median; M500, malathion 500 mg/mL; M1000, malathion 1000 mg/mL. TBARS, Thiobarbituric acid reactive substance; MDA, Malondialdehyde; GST, Glutathione-S-Transferase; GSH, Reduced glutathione; CAT, Catalase; SOD, superoxide dismutase.



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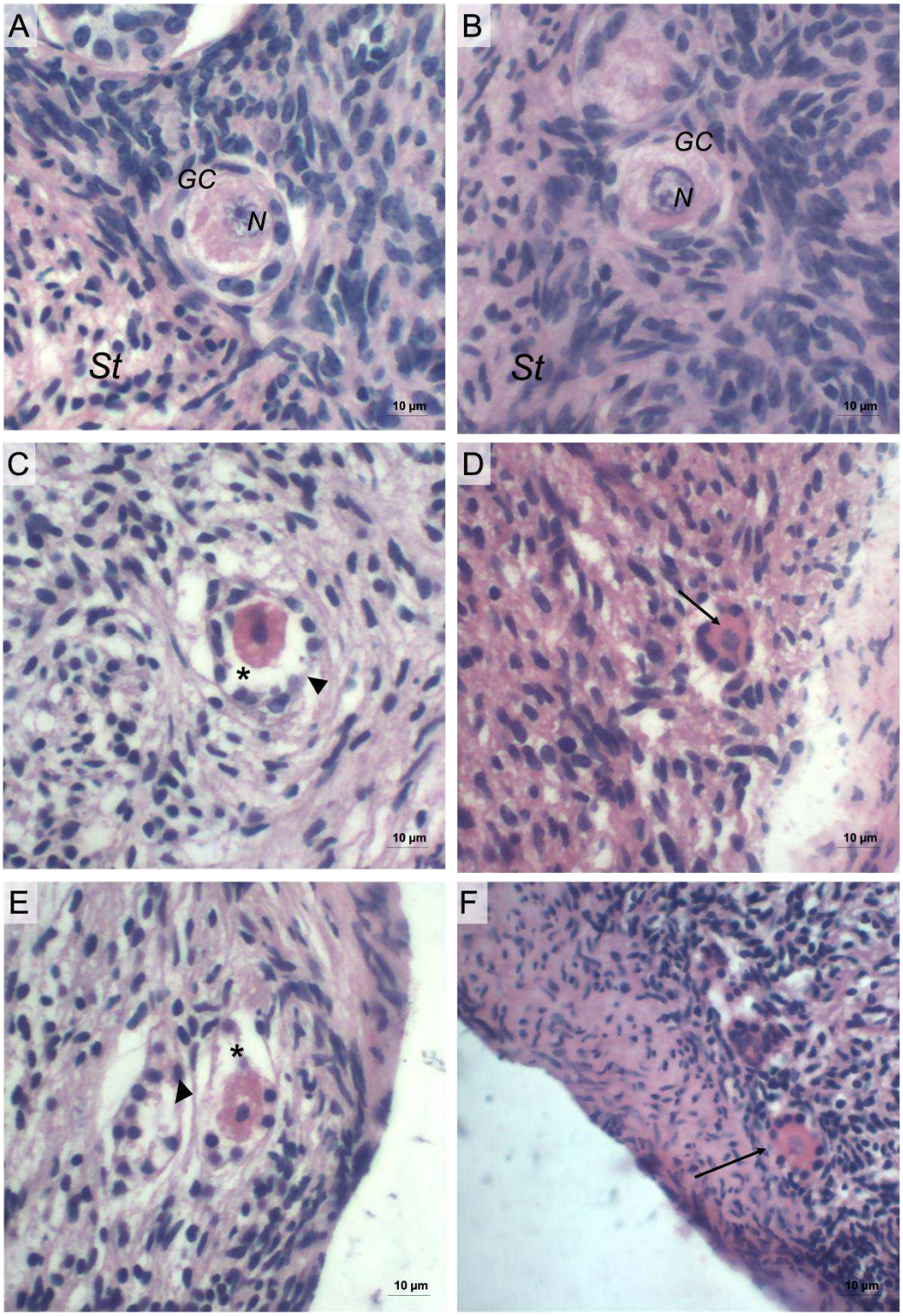

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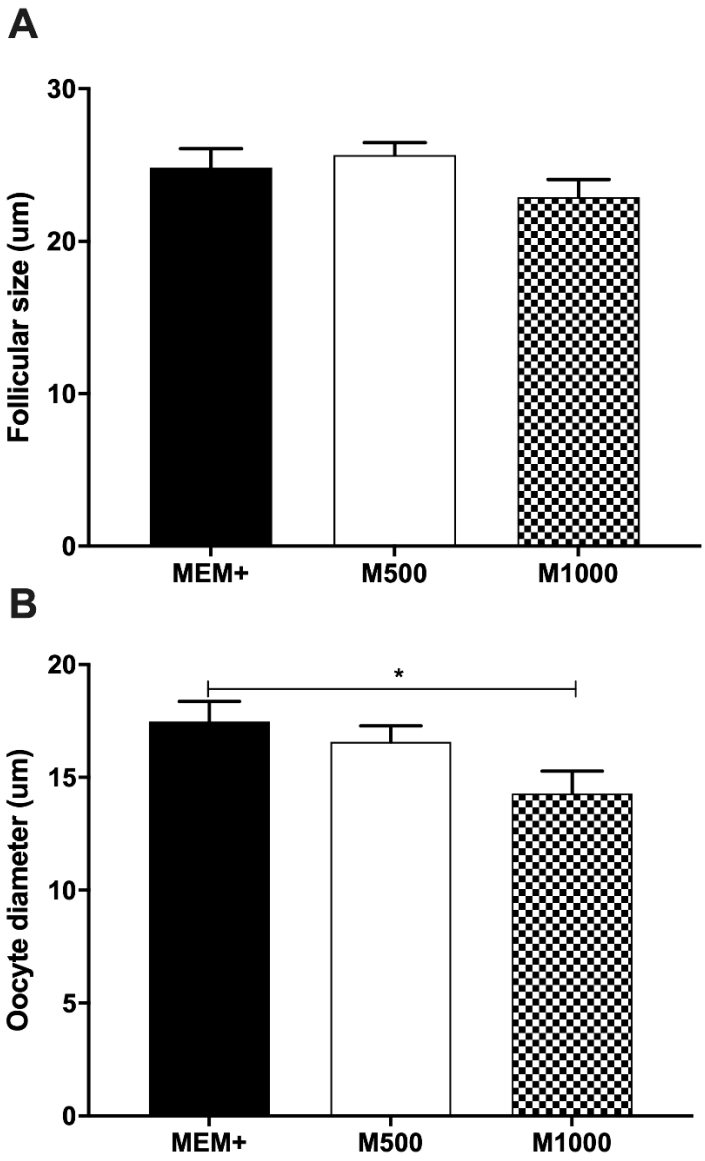

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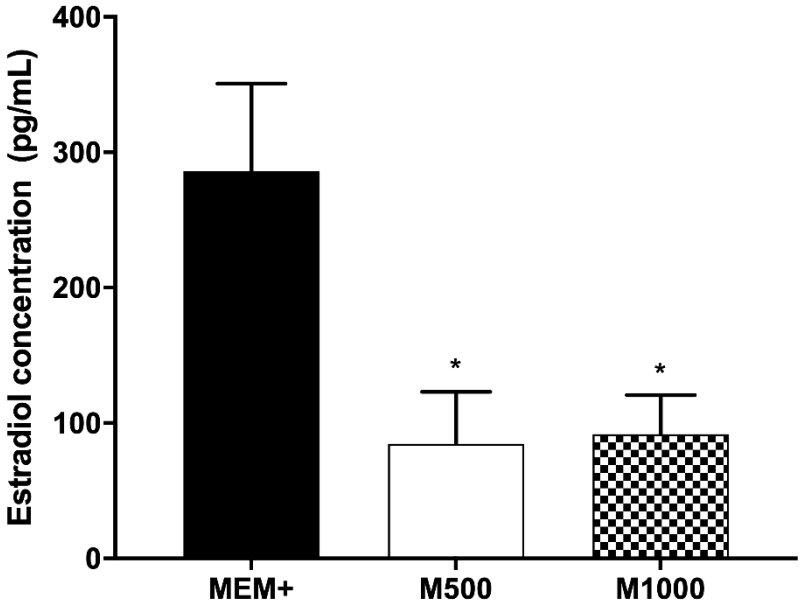

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7 ARTIGO 4

Low dose of Malathion impairs ovarian, uterine and follicular integrity by altering oxidative profile and gene expression of rats exposed during the peripubertal period

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Low dose of Malathion impairs ovarian, uterine and follicular integrity by altering oxidative profile and gene expression of rats exposed during the peripubertal period

Rafaela Pires Erthal[1,2], Giovanna Fachetti Frigoli[1], Ariana Musa de Aquino[3], Ana Paula Franco Punhagui[1,2], Wellerson Rodrigo Scarano, [3] Glaura Scantamburlo Alves Fernandes[1*]

1 – Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970
2 – Department of General Pathology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970
3 – Department of Morphology, São Paulo State University – UNESP, Botucatu, Sao Paulo, Brazil, Postal code: 18618-689

***Corresponding author**
Glaura Scantamburlo Alves Fernandes
Tel.: +55 43 33714417
E-mail address: glaura@uel.br (G.S. A. Fernandes)

Abstract

Malathion is an important pesticide used in agriculture and control of the *Aedes aegypt* mosquito. Since there are no studies evaluating the damage mechanisms of malathion on the female reproductive system, the aim of the study was to evaluate whether exposure of female rats to low doses of malathion during the juvenile and peripubertal periods can compromise the onset of puberty, estradiol levels, ovarian and uterine integrity and mechanisms involved in damage. For this, thirty juvenile female rats were exposed to vehicle or malathion (10 mg/kg or 50 mg/kg) between postnatal days 22 and 60 and the onset of puberty verified. At the end, blood was collected for estradiol dosage. Ovary and uterus were destined for evaluation of histological integrity, oxidative stress and expression of genes related to cell proliferation, antiapoptotic and endocrine pathway. Although estradiol levels and the onset of puberty were not disrupted, malathion exposure compromised ovarian and uterine integrity and morphometry through altered oxidative profiles and genes related to the regulation of cell-cycle, anti-apoptotic and endocrine pathways. Our data point to the role of induction of cell proliferation and survival and oxidative damage by malathion on the female reproductive system of exposed rats during peripubertal periods.

Keywords: malathion, postnatal development, oxidative stress, gene expression, uterus, ovarian follicles

1. Introduction

Infertility is a disease of the female or male reproductive system that impairs reaching pregnancy after 12 months of regular intercourse. According to the World Health Organization (WHO), infertility affects an estimated 48 million couples. Female infertility can occur mainly due to abnormalities of the ovaries, fallopian, uterus and endocrine system (WHO, 2020). While the main factors thought to impair fertility are an increase in the age of conception in women, the presence of sexually transmitted infections (STIs), and emotional and physical stress (Hart, 2016), other factors such as lifestyle and environmental contaminants are rarely addressed and can also play an important role in this process (Borght and Wyns, 2018).

Since the beginning of the use of pesticides as a form of pest control in agriculture or other forms of disease transmission, it has become increasingly necessary to use such compounds, especially for large-scale food production. Exposure to environmental contaminants, heavy metals, cosmetic products, among others, is becoming more and more frequent among different populations. Such exposure factors may be directly related to cases of idiopathic infertility (Yazar, 2022).

Malathion is one of the important pesticides of the organophosphate class widely used worldwide in agricultural cultivation and control of the *Aedes aegypt* mosquito. The compound acts by inhibiting the action of the enzyme acetylcholinesterase, resulting in the overactivation of the cholinergic pathways (Selmi et al., 2018). According to Environmental Protection Agency (EPA), more than 30 million pounds of malathion are used per year in cultures of cotton and rice (EPA, 2004). The widespread use of malathion leads to environmental contamination and an increase in the number of people exposed to the compound (Navarrete-Meneses et al., 2017), especially in developing countries (Varol et al., 2015). The World Health Organization allows a daily intake of 0.02 mg/kd/day of malathion through food contamination (WHO, 2007). Even if people are exposed to low doses of malation, prolonged exposure can compromise the different systems of the human body (Badr, 2020; Zidan, 2015).

Malathion exerts its main toxicity mechanisms in humans through the induction of oxidative stress, and can damage DNA integrity and induce apoptotic pathways (dos Santos et al., 2016). Previous studies by our group have shown that male rats exposed to low doses of malathion during critical periods of development showed morpho-physiological impairment in testes, epididymides and spermatozoa. We further showed that this damage in the male reproductive system was related to changes in oxidative profile and hormonal dysregulation.

However, there are no studies that better reveal the effects of exposure of female rats on reproductive parameters and possible mechanisms involved during critical periods for the postnatal development of this system: the juvenile and peripubertal periods. Studies using an exposure model of

adult rats to malathion (33 and 100 mg/kg) showed that there was impairment of ovarian tissue and an increase in the number of atrophic follicles especially through impairment of the activity of the antioxidant enzymes, SOD and catalase (Koc et al., 2009a; Ozsoy et al., 2016). Other authors relate the damage observed in ovarian tissue to induction of apoptotic markers and decrease in markers for cell proliferation (Madiha et al., 2011; Yong et al., 2021). On the other hand, study have shown that malathion (170 mg/kg) favored mammary gland proliferation and adenocarcinoma development after exposure of rats for 28 days (Cabello et al., 2001).

Since there are no studies addressing exposure of rats to low doses of malathion during the postnatal development period of the female reproductive system, we chose to address exposure of these animals during the juvenile and peripubertal periods according to Ojeda et al. (1980). During this period, the hypothalamic-pituitary-gonadal axis will be established, involving the development and maturation of the female genital system to (OJEDA et al., 1980). During this period, puberty will be installed, evidenced in the females by the vaginal opening and the beginning of the estrous cycle, approximately between postnatal days (PND) 30 and 37 (Laffan et al., 2017). We further point out that disruption by external agents during critical periods for the development and maturation of the female reproductive system can compromise reproductive health and even manifest itself in adulthood (Angel Sánchez-Garrido et al., 2022).

From this, the aim of the present study was to evaluate whether exposure of female rats to low doses of malathion, according to the literature, during the juvenile and peripubertal periods can compromise the onset of puberty, ovarian and uterine integrity, follicular morphophysiology and possible mechanisms involved in damage.

2. Materials and methods

2.1. Animals and experimental conditions

Thirty juvenile female Wistar rats from different litters at postnatal day 21 (PND21) were supplied by the Animal House of Biological Sciences Centre, State University of Londrina (CCB-UEL) and were acclimated to the new environment at the Laboratory of Toxicology and Metabolic Dysfunction of Reproduction for a day before the beginning of the experimental period. Animals were kept under recommended conditions at the local animal house. They were allocated into polypropylene cages (43 x 30 x 15 cm) (3 animals/cage) with laboratory-grade pine shavings as bedding during the entire experiment. Temperature and lighting were controlled (~ 23°C; 12L, 12D photoperiod, lights switched off at 07:00 pm). Rat chow and filtered tap water were provided *ad libitum*. Animal care and

handling procedures were in accordance with the National Institutes of Health Guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and with the approval of the Ethics Committee on Animal Use of State University of Londrina (CEUA/UEL protocol number Of. CIRC CEUA n. 01/2020).

2.2. Experimental design

Animals were randomly distributed in three experimental groups of 10 animals each: control (C), malathion 10 mg/kg body weight (b.w.) (M10) and malathion 50 mg/kg b.w. (M50). Malathion was used at doses lower than the subchronic no observed adverse effect level (NOAEL) dose (130 mg/kg b.w.) for the rat's reproductive system in relation to developmental toxicity (FAO, 1997). The average doses used in this study represents the adjustment (Foureman and Kenyon, 2006) of the AOEL dose of 0.03 mg/bw/day in humans (European Commission). Considering intraspecies variability we added a security factor of 10 (Nielsen and Grete Ostergaard, 2008). These doses are considered low and relatively safe to reproductive parameters by previous studies (Welshons et al., 2006), and have been used in our group previous studies using male Wistar rats (R. P. Erthal et al., 2020; Rafaela Pires Erthal et al., 2020).

Malathion doses were administered in accord to Geng et al. (2015), that reported reproductive disorders in Wistar rats exposed to 54 mg/kg b.w. malathion during adult life. In the present study, and in our group's previous studies (R. P. Erthal et al., 2020; Rafaela Pires Erthal et al., 2020), the experimental period was adjusted to PND 22 to 60, reaching the juvenile and peripubertal periods, stablished according to Ojeda et al. (1980). Animals were exposed orally *via gavage* to 10 or 50 mg/kg b.w. Saline 0.9% was used as vehicle. All groups were treated daily during the experimental period and the animals in control group received only the vehicle.

2.3. Preparation of malathion solution

Malathion (dietil-dimetoxitiofosforiltio; CAS no. 121-75-5; Cheminova) was obtained from Dominus Quimica (Jandaia do Sul, Brazil). The compound was diluted daily in 0.9% saline.

2.4. Determination of puberty installation

The average day of complete vaginal opening was determined. In this day, rats were weighted and vaginal lavage were collected daily as described by Marcondes (2002), in order to determine the

day of the first estrus, characterized by the predominance of cornified epithelial cells. The first estrus and occurrence of the vaginal opening are indicatives of puberty installation in female Wistar rats. Observations were made starting at PND 30.

2.5. Materials collection

At the end of the experimental period, during the estrus phase, rats were intraperitoneally anaesthetized with a combination of ketamine 75 mg/kg b.w. (Sedomin® 10%, Avellaneda, Argentina) and xylazine 10 mg/kg b.w. (Anasedan®, Paulínia, Brazil), weighed and euthanized by cardiac puncture. Blood was collected in heparinized tubes for hormonal dosages. The right ovaries and right uterine horn were harvested, weighted and fixed in Alfac solution (85% alcohol 80%, 10% formaldehyde and 5% acetic acid). The organs were embedded in Paraplast® (SIGMA Life Science). Three non-consecutive sections (5um thick) per animal separated by 100 um distance were obtained, mounted on glass slides and stained with haematoxylin and eosin (HE). The sections were examined under light microscopy for general histopathological and morphometric analysis. Left ovaries and left uterine horn were collected and stored in –80ºC freezer and destined for evaluation of the oxidative profile and gene expression of ER-α/ER-β, BCL-2, TP53, SLUG e β-catenin via RTq-PCR.

2.6. Oestradiol dosage

Blood plasma was obtained via centrifugation at 2400 rpm for 20 min at 3,5 °C and stored at −20 °C until hormonal assay. Hormonal assay were performed in the Laboratory of Applied Immunology in the State University of Londrina's Hospital for identification of plasma oestradiol concentration. All samples were dosed in the same assay, to avoid inter-assay variations.

2.7. Morphological analysis of ovaries and uterus

The number of corpora luteum present in a histological cut of each animal was counted. Ovarian follicles classification was made according the different stages of follicle development, follicle morphology and the number of follicular cells layers (Borgeest et al., 2002; Talsness et al., 2005). Follicles were classified as: primordial when presented an oocyte surrounded by a single layer of flattened follicular cells; primary when presented an oocyte surrounded by a single layer of cuboidal follicular cells; secondary when presented two layers of cuboidal follicular cells; antral when they presented many layers of granulosa cells and a liquid filled cavity surrounding the oocyte.

For evaluation of the diameter of the oocytes and follicles forty random cross-sections in ten different images per animal captured with an image system attached to the microscope (Motic, Xiamen, China) were examined. The diameters of both structures were measured using Motic Image Plus 3.0 (Motic, Xiamen, China). In each oocyte or follicle, the mean of four measures for the diameters was calculated and used in the statistical analysis.

The parameters observed in the uterus were the height of the endometrium, myometrium and perimetrium, and the height of the luminal and glandular epitheliums. Also, the number of glands per cut in the uterus was counted. In each animal, 10 different regions were analysed in which were made four measurements also using Motic Image Plus 3.0 (Motic, Xiamen, China). For each parameter evaluated, it was used a total of forty measurements per animal.

2.8. Oxidative profile evaluation of ovaries and uterus

2.8.1. Tissue preparation

The frozen fragments were homogenized with a Ultraturrax homogenizer (Marconi) in Phosphate Buffered Saline (pH 7.2) and normalized to 1 mg/mL protein concentration according to the Bradford method (Bradford, 1976). The homogenate was used to determine the levels of thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST), reduced glutathione (GSH), catalase, and superoxide dismutase (SOD).

2.8.2. Lipoperoxidation

The TBARS levels lipid peroxidation as described previously by Federici et al. (2007). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, was determined by the difference between absorbance at 535 and 572 nm (Multiskan GO, Thermo Scientific, Vantaa, Finland). The results are expressed as nanomoles of MDA per milligram of protein.

2.8.3. Glutathione S-transferase

The activity of glutathione S-transferase was evaluated by spectrophotometry using GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and potassium phosphate buffer. Sample absorbance was measured at 340 nm at 40 sec intervals during 5 min, according to Keen et al. (1976).

2.8.4. GSH measurement

Reduced glutathione (GSH) levels were determined according to the method proposed by (Rahman et al., 2007), with some modifications, using 5,5'-dithiobis 20-nitro benzoic acid in the homogenate, as evidenced by the yellow colour formation 26. GSH levels were measured at 412 nm, and the results were expressed as micromoles/mg of protein.

2.8.5. Catalase activity assay

Catalase activity was determined based on the principle of peroxide dismutation. The absorbance was measured at 240 nm at 15 sec intervals for 1 min 27.

2.8.6. Evaluation of SOD activity

The evaluation of SOD activity was performed as per the method described by Senthilkumar et al. (2021). Homogenates normalized to 1 mg/mL were used as the enzyme source. A reaction mixture containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazolium (NBT) (96 µM), and Triton X-100 (0.6%) was prepared and incubated for 2 min with 20 mM hydroxylamine hydrochloride (NH2OH· HCl) at pH 6.0. The final volume was adjusted to 200 µL. The reaction consists of quantifying the complex formed between superoxide anions by the addition of NBT and NH2OH· HCl with yellow coloration and the reduction of NBT, forming a blue colour reading at 560 nm. The samples were read every 15 s for 2 min.

2.9. Evaluation of gene expression by Real-Time Polymerase Chain Reaction after Reverse Transcription (RT-qPCR)

Gene expression in ovaries or uterus was evaluated by RT-qPCR related to cell survival, proliferation and death and hormone receptor. RNA extraction was performed with the Trizol kit (Ambion, USA) according to the manufacturer's instructions. RNA was quantified by spectrophotometry using the NanoVue equipment (GE Healthcare Life Sciences, USA). The analysis of RNA quality was obtained by the RNA integrity number (RNA Integrity Number, RIN), from the analysis of ribosomal RNAs based on microfluids, using the 2100 Bioanalyzer system (Agilent, USA) (Becker et al., 2010; Fleige and Pfaffl, 2006). The mRNA Reverse Transcription Reaction was performed using the High

Capacity RNA-to-cDNA Master Mix kit (Life Technologies, USA), following the manufacturer's guidelines. For the reaction, 4 μL of Master Mix for reverse transcription was used, to which 1 μg of RNA was added and the volume made up to 20 μL with nuclease-free water. The mixture was incubated under the following conditions: 25 °C for 5 min., 42 °C for 30 min. followed by reverse transcriptase inactivation at 85 °C for 5 min. For each RT-qPCR reaction for mRNAs, 10 μL of GoTaq® qPCR Master Mix, based on SYBR Green chemistry (Promega, USA), 5 μL of the RT reaction and 1 μL of "sense" and "antisense" primers were used at 10 μM and the volume was made up to 20 μL with nuclease-free water. Primers for the genes were designed using the Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Thermocycling was performed in the QuantStudio equipment (Life Technologies, USA), under the following conditions: GoTag Hot Start Polymerase activation 2 min. at 95°C followed by 40 cycles of 15 sec. at 95°C and 1 min. at 60ºC, finally, dissociation curve in the range of 60-95°C. An amplification graph was plotted for each sample showing an increase in fluorescent reporter dye (ΔRn) in each PCR cycle. From this graph, the cycle where the reaction crosses the detection threshold (cycle threshold - CT) was determined. The relative quantification of each gene was performed using the 2-ΔΔ CT method according to Livak and Schmittgen (2001). The values obtained for all samples were normalized by the ratio obtained between the target genes and the endogenous gene (Table 1).

3. Results

3.1 Exposure to malathion did not affect anthropometric parameters, estradiol level and onset of puberty

Data regarding anthropometric parameters, estradiol level and onset of puberty are presented in Table 2. Our experimental design showed that female rats exposed to low doses of malathion during the juvenile and peripubertal periods had no changes in body, uterine and ovarian weight when compared to the control group. The same is observed in estradiol levels, which did not differ between the experimental groups. Regarding the onset of puberty, the mean day of vaginal opening and first estrus did not differ between the experimental groups.

3.2 Low doses of malathion during juvenile and peripubertal period impaired ovarian morphophysiology

Although follicular development did not change after exposure to malathion, as shown in Table 3, both doses increased the percentage of abnormal follicles compared to the control group. In addition,

the M10 group had a smaller oocyte size while the M50 group had a smaller follicular size compared to the control group after exposure to malathion. These data are presented in Table 3.

3.3 Low doses of malathion during the juvenile and peripubertal periods compromise uterine development

The uterine morphometric evaluations, presented in Table 4, show that only malathion 10 mg/kg led to an increase in myometrium thickness, while only the 50 mg/kg dose induced a decrease in perimetrium and an increase in glandular epithelium in relation to group control. In addition, both doses increased the thickness of the luminal epithelium compared to the control group. There was no significant difference between the groups regarding the parameters of endometrial thickness and number of glands.

Uterine histopathology analysis showed that both doses impaired tissue integrity, as shown in Figure 1. Cell desquamation in the glandular lumen, proliferative epithelium and polymorphism of glandular epithelium were observed, as well as the presence of vacuole after both exposures to the compound. Furthermore, vascular congestion was observed in vessels in the M50 group.

3.4 Malathion at low doses compromises the oxidative profile and activity of antioxidative enzymes in both ovaries and uterus of female rats exposed during the juvenile and prepubertal periods

The contradictory effects of malathion on oxidative parameters are shown in Table 5 for ovaries and Table 6 for uterus. While in the ovaries there was an increase in lipid peroxidation after exposure to both doses of malathion, a reduction in LPO levels was observed in the M50 group compared to the control group in uterus. No change was observed in the M10 group.

When evaluating the antioxidant profile in ovaries, we observed that there was no change in the levels of GSH or in the activity of the catalase enzyme between the experimental groups (Table 5). On the other hand, there was a reduction in glutathione-S-transferase enzyme activity levels in both malathion groups compared to the control group, in addition to a reduction in SOD enzyme activity after exposure to malathion 50 mg/kg.

Similar to the observed in the ovaries, the levels of GSH and catalase activity in the uterus did not change, regardless of the exposure. In the same way, both doses of malathion reduced SOD enzyme activity and increased glutathione-S-transferase enzyme activity.

3.5 Alteration of expression of genes related to hormone and cell cycle regulation in ovary and uterus after malathion exposure in females

The expression of genes in ovaries is shown in Figure 2. Exposure of females to low doses of malathion did not significantly alter the expression of β-catenin, FGF2 and TP53 genes. Both doses of malathion increased gene expression of ER-β and Slug mRNA. On the other hand, the lowest dose of malathion (10 mg/kg) was the only one to increase the expression of genes related to cell regulation, namely Bcl-2 and FOXO in relation to the control group. The M50 group showed no changes in the expression of these genes.

The uterine tissue response to gene expression was different from ovaries, as shown in Figure 3. There was no change in the expression of FGF-2 and FOXO genes. On the other hand, both doses of malathion were sufficient to significantly increase mRNA expression for ER-α, β-catenin, Bcl-2, Slug, and TP53 genes relative to the control group.

4. Discussion

In the present work we observed that although low doses of malathion did not compromise estradiol level or puberty onset, the exposure was sufficient to impair the integrity and morphology of ovaries and uteri in female rats. Such changes are justified through disruption of the oxidative profile and alteration in the expression of genes related to the hormonal pathway or regulation of cell proliferation.

It is well established that increased reactive oxygen species cause tissue damage through ATP depletion, DNA damage, protein damage, and lipid membrane damage (Behrman et al., 2001). Literature shows that oxidative damage is one of the main mechanisms in impairing ovarian and uterine health, favoring disorders of the female genital system such as polycystic ovary syndrome (Hyderali and Mala, 2015) and endometriosis (Da Broi and Navarro, 2016). The increased number of atresic follicles observed in the present study correlates with the increased MDA levels after exposure to both doses of malathion.

Follicular atresia is a physiological process that involves the apoptosis of most follicles that start growing but do not reach the pre-ovulatory stage (Hsueh et al., 1994; McGee and Hsueh, 2000). The absence of stimuli such as the gonadotropins - LH and FSH, estradiol, growth factors and cytokines direct the depletion of granulosa cells and consequently follicular degeneration through activation of apoptosis. Finally, the cells are then eliminated (Matsuda et al., 2012). However, in pathological situations, oxidative stress-induced tissue destruction itself can compromise follicular integrity and induce follicular atresia (Behrman et al., 2001), as observed in the present study.

The increased MDA levels observed in the ovary are directly related to the decreased activity of important antioxidant enzymes, SOD and GST. We emphasize that oxidative stress results from an

imbalance between oxidizing free radicals and antioxidant species (Melchiorri et al., 1996). The enzymes GSTs and SOD are important in the detoxification of ROS, prevent the excessive formation of free radicals and the damage caused by them. A disturbance in their activities directly stimulates oxidative stress (Gusti et al., 2021; Kalender et al., 2010).

Our results corroborate other studies showing that malathion exposure in different experimental models impaired ovarian integrity through increased free radicals and decreased activity of antioxidant enzymes, SOD and catalase (Arab et al., 2018; Bhardwaj and Saraf, 2016; Koc et al., 2009b; Yong et al., 2021). Unlike the studies pointed out above, in our study no change in GSH levels or catalase activity was observed at the doses discussed.

We note here that there was no change or decrease in follicular development at the expense of an increase in atrophic follicles. This therefore confirms that the increase in atresic follicles is probably due to oxidative damage observed in the ovaries and not alteration in follicular development. This result is also supported by the increased expression of anti-apoptotic genes, Slug and Bcl-2 (Maji et al., 2018), which allowed the atrophic structure to remain. In this case, the increased expression of these genes favors cell survival and the permanence of atrophic follicles, without their being recognized and then eliminated.

In addition to the increased expression of genes involved in the modulation of apoptosis, there was also increased expression of genes that favor cell proliferation regulation and development, namely ER-b and FOXO. FoxO is a member of the forkhead transcription factor family that plays an important role in cell cycle regulation, DNA damage repair, apoptosis, and oxidative stress (Zhang et al., 2020). Zhang et al. (2020) points out that the expression of this gene is involved with the physiological regulation of follicular development and the progression of ovarian-related diseases. Overexpression of FOXO can regulate follicular development, increase follicular reserve and ovarian reproductive capacity (Castrillon et al., 2003; Pelosi et al., 2013). The data agree with the one observed in the present study, as follicular development and ovarian reproductive capacity was maintained concomitant to upregulation of the FOXO gene.

In addition, the overexpression of the gene possibly favored the increase in oocyte and follicle size by increased follicular reserve. Increased follicle size was also observed after exposure of adult female rats to chlorpyrifos (2.5 mg/kg), an organophosphate, for 8 weeks (Nishi and Hundal, 2013). We emphasize that medium-sized oocytes have better fertilization and subsequent embryonic development rates than small or large oocytes. Thus, the increased oocyte and follicle size observed in the present study may impair fertilization and embryo development (Wirleitner et al., 2018).

Both FOXO and ER-ß, are involved in tumor suppression by cell cycle inhibitor activity (Hedrick, 2009; Paik et al., 2007) and in the endocrine signaling pathway. The expression of FOXO has been implicated in the regulation of genes involved in steroid biosynthesis (Z. Liu et al., 2009), while that of ER-b in the action of estrogens by acting as ligand dependent transcription factors (Auwerx et al., 1999).

The FOXO gene responds to oxidative stress, justifying a possible mechanism that led to the increased expression of this gene observed in the present study (Lim et al., 2017; Wang et al., 2017). In turn, the FOXO gene was noted to stimulate the regulation of ERs genes (Madureira et al., 2006), again corroborating the present study. Thus, we suggest that the oxidative damage observed in the ovarian tissue of rats exposed to malathion induced FOXO overexpression and, this, ER expression.

Although the follicular size was increased, its function of estradiol production was not impaired and normal levels were maintained. This can be justified by the fact that the expression of receptors for steroid hormones (ER-b) is increased and therefore increasing the sensitivity of the tissue to the hormone. This also justifies why puberty installation was not altered and organ weights were maintained after exposure of peripubertal rats to malathion.

About the uterus, we also observed alteration in the oxidative profile, but in a totally different way. We emphasize that there are no studies that address the impact of malathion on this tissue. Abolaji et al. (2015) also observed this different response in the oxidative profile between ovary and uterus after exposure to 2,5-hexanedione, an industrial chemical. After rats were exposed to the compound, although decreased activity of the antioxidant enzymes SOD and GST was observed in the ovary, the compound induced increased activity of these enzymes in the uterus, corroborating our study and showing tissue-dependent oxidative responses. The increased uterine SOD and GST activity may be the result of an adaptive response in order to combat elevated levels of oxidative stress (Abolaji et al., 2015). However, after these increased, the enzymes exert their effects neutralizing and decreasing oxidative species, manifested in the reduced lipoperoxidation in the present study after rats exposed to malathion. The same effect of increasing antioxidant enzymes was observed by Mondal et al. (2018), which led to reduced MDA levels compared to the control group after stimulation by monosodium glutamate.

It is worth noting that although in PND60 the decrease in MDA levels was observed in the present study, the change in the antioxidant profile shows oxidative stress probably occurred in an earlier period. Previous study also showed that malathion exposure (20 ppm) of rats for 4 weeks increased the levels of the enzymes SOD and GST in serum, as well as MDA levels (Ahmed et al., 2000). It also highlights that increased activity of these enzymes can efficiently scavenge toxic free radicals and partially protect against lipoperoxidation caused by malathion exposure. Studies have already pointed to oxidative stress as a biomarker (Coutinho et al., 2019) for uterine disorders, ranging from endometriosis (Samimi et al., 2019) to cancer (Kajiyama et al., 2019).

The impairment in the oxidative profile observed in our study is consistent with the fact that uterine integrity was severely impaired after exposure to both doses of malathion, as evidenced by shedding of uterine cells and the presence of vacuoles in the luminal epithelium. Keys and King (1989) showed that the presence of degeneration and vacuoles in the uterine epithelium stems from dysfunction of the epithelial junctions or cell death. Damage to the integrity of the luminal epithelium and endometrial glands compromises concept survival and growth in peri-implantation phases (Gray et al., 2002).

The presence of vacuoles can be observed in the physiology of the menstrual cycle of the endometrium in humans. These vacuoles are mostly loss of epithelial cells with formation of apoptotic bodies in the secretory, premenstrual and menstrual phases (Hopwood and Levison, 1976; Spornitz et al., 1994). However, the increase in vacuoles may represent an pathological overactivation of apoptosis pathways (Elgamal et al., 2016)

Apoptosis plays an important role in tissue maintenance since it allows the removal of excess or damaged cells. The balance between apoptosis and cell proliferation is essential for tissue maintenance. Specifically in the uterus, apoptosis allows the removal of cells during menstruation (Samimi et al., 2019). On the other hand, a reduction in cell death in the uterine endometrium may favor uterine hyperplasia with a higher survival rate of these cells (Nishida et al., 2005).

In the present study, we observed that important anti-apoptotic enzymes, called Bcl-2 and slug, were overexpressed, thus enabling cell survival. Concomitantly, there was an increase in genes involved in stimulating endometrial cell proliferation, namely ß-catenin (Corachán et al., 2019) and ER-α (Kao et al., 2002; Salmi and Rutanen, 1996). Activation of ER-a by estrogen stimulates endometrial hypertrophy and hyperplasia, increasing the risk of the epithelium becoming neoplastic (Lessey et al., 2006). Together, these genes stimulate cell proliferation and survival. On the other hand, there was also an increase in the expression of TP53, the important DNA guardian gene, which induces cell cycle arrest for DNA repair and, if necessary, apoptosis (Guimaraes and Hainaut, 2002). So, expression of this gene prevents tumorigenesis (Taylor et al., 2006). Here we can observe an attempt by the tissue to try to regulate and maintain the homeostasis of cell proliferation.

From the observation of the uterine histopathology, we noticed that the increase in TP53 was not enough to prevent the action of the overexpression of the other pro-proliferative and anti-apoptotic enzymes. This statement is supported by the increased size of the luminal epithelium and myometrium. Probably, in a compensatory manner, there was a decrease in the perimetrium and glandular endothelium, since there was no change in uterine weight. Yu et al. (2013) showed that maternal exposure to a mixture of organophosphates induced endometrial thickening and hyperplasia in first generation rats. Corroborating the study, we observed both hyperplasia of the luminal epithelium and thickening of this endometrial layer.

In addition to increased endometrial thickness, we also observed increased myometrial size after exposure to malathion. Although no tissue abnormalities were found, hyperplasia of the uterine muscle layer is commonly associated with benign tumors (Wilkinson and Rollason, 2001). Cabello et al. (2001) reported that rats (PND 16) exposed twice daily for 5 days to malathion (170 mg/kg) subcutaneously or intraperitoneally showed alteration in the epithelium of the mammary glands, favoring the process of carcinogenesis.

In the present study, we observed that there was uterine hyperplasia mediated by overexpression of proliferative genes and tissue damage through disruption of the oxidative profile. However, we emphasize that at this point, no neoplastic figures were observed. The question remains: could exposure

to such compounds during life favor uterine carcinogenesis? The initial stimulus already exists by malathion during the early periods of life.

5. Conclusion

From the experimental model, we concluded that exposure to low doses of malathion during the juvenile and peripubertal periods was not sufficient to impair the onset of puberty or estrous level but impaired the integrity and morphometry of ovarian follicles as well as of the uterine endometrium. This damage occurred in the organs through the altered oxidative profile manifested by levels of lipoperoxidation and antioxidant species, as well as altered gene expression of genes involved in cell proliferation, anti-apoptotic pathways and endocrine regulation. Furthermore, we point out that the harm of in vivo exposure of rats to malathion was shown to be organ-dependent since the ovary and uterus behaved differently to the stimulus, especially when it came to altered gene expression. These findings combined show that malathion compromised the reproductive health of exposed rats during critical periods of development.

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Table Legends

Table 1: List of Designated Primers for RTq-PCR

Table 2. Effects of juvenile and peripubertal exposure to low doses of malathion on body and organ weight parameters, food and water consumption, estradiol levels and onset on puberty
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 3. Effects of juvenile and peripubertal exposure to low doses of malathion on histological parameters of ovary
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01; ***p>0.001. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 4. Effects of juvenile and peripubertal exposure to low doses of malathion on histological parameters of uterus
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01; p<0.001; ****p>0.0001. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 5. Effects of juvenile and peripubertal exposure to low doses of malathion on oxidative profile of ovary
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 6. Effects of juvenile and peripubertal exposure to low doses of malathion on oxidative profile of uterus
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Figure legends

Figure 1. Effects of juvenile and peripubertal exposure to low doses of malathion on uterus histopathology

Photomicrographs of uterus sections from the control (A-C), M10 (D-I) and M50 (J-O) of malathion groups. Control animals reveals normal appearance of luminal (A and B) and glandular (C) epithelium, and normal stroma of the uterine mucosa (A and B). (E,F,K and L) Desquamated cells in glandular lumen (asterisk). (G and M) Proliferative epithelium (thick arrow) and presence of vacuole in the epithelium (thin arrow). (H and L) Glandular epithelial polimorfism (arrowhead). (I, O) Inflammatory cells in (i.c.) and (N) vascular congestion (v.c.). M10 - rats treated with 10 mg kg−1 malathion; M50 - rats treated with 50 mg kg−1 malathion.End, endometrial stroma; l, lumen; ee, epithelial endometrium; ge, glandular epithelium. Haematoxylin and eosin stain (A,D,J: 40X magnification; B,E,K: 100X magnification; C, F-I, L-O: 400X magnification).

Figure 2. Effects of juvenile and peripubertal exposure to low doses of malathion on ovarian mRNA expression of (A) ER- β, (B) β-catenina, (C) Bcl-2, (D) FGF-2, (E) FOXO, (F) Slug and (G) TP53.

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Figure 3. Effects of juvenile and peripubertal exposure to low doses of malathion on uterine mRNA expression of (A) ER-α, (B) β-catenina, (C) Bcl-2, (D) FGF-2, (E) FOXO, (F) Slug and (G) TP53.

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 1: List of Designated Primers for RTq-PCR

Gene	Forward primer	Reverse primer
GAPDH	GCTCTCTGCTCCTCCCTGTTC	GAGGCTGGCACTGCACAA
ERα	CACATCAGGAAATGTCAAGCAGT	AAGAGCTAAGCCAGTCGCTC
ERβ	TGAGTGCAGCTCAACAGAGG	TCTGTAGTCTGTCCGCCTCA
BCL2	ACTCTTCAGGGATGGGGTGA	TGACATCTCCCTGTTGACGC
FOXO	CTTTCCCGTGGAGCAGAACT	GTGCTCTGGAGTAGGGATGC
TP53	TCATGGAGGATTCACAGTCGG	TCGCTGTGGTGGGCAGAATA
FGF-2	TCCATCAAGGGAGTGTGTGC	TCCGTGACCGGTAAGTGTTG
Slug	CCTCATCTTTGGGGCGTGTA	ATGGCATGGGGGTCTGAAAG
b-catenina	ACTCCAGGAATGAAGGCGTG	GAACTGGTCAGCTCAACCGA

Table 2. Effects of juvenile and peripubertal exposure to low doses of malathion on body and organ weight parameters, food and water consumption, estradiol levels and onset on puberty

	Experimental groups		
Parameters	**Control (n=10 animals)**	**M10 (n=10 animals)**	**M50 (n=10 animals)**
Body weight (g)			
Initial	52.4 ± 1.28	50.6 ± 1.65	49.6 ± 1.41
Final	185.3 ± 7.68	182.6 ± 4.95	180.1 ± 6.33
Organ weight (g)			
Ovary	0.06 ± 0.004	0.06 ± 0.007	0.06 ± 0.002
Uterus	0.54 ± 0.034	0.44 ± 0.091	0.52 ± 0.05
Estradiol concentration (pg/mL)	81.4 ± 5.87	77.3 ± 14.37	67.7 ± 10.23
Onset of puberty (postnatal day- PND)			
Vaginal opening	31.0 ± 0.33	31.7 ± 0.26	31.5 ± 0.34
First estrus	34.6 ± 1.00	35.0 ± 0.79	35.3 ± 0.83

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 3. Effects of juvenile and peripubertal exposure to low doses of malathion on histological parameters of ovary

	Experimental groups		
Parameters	**Control (n=5 animals)**	**M10 (n=5 animals)**	**M50 (n=5 animals)**
Ovarian follicles count (%)			
Primordial/Primary	75.7 ± 1.5	78.8 ± 4.3	77.5 ± 1.3
Preantral	6.9 ± 2.4	4.3 ± 1.1	3.8 ± 0.1
Antral	17.4 ± 1.5	16.9 ± 3.3	18.7 ± 1.4
Corpus luteum number	5.0 ± 0.6	4.5 ± 0.8	3.2 ± 1.0
Morphometric parameters (µm)			
Follicle size	28.7 ± 1.0	29.7 ± 1.2	34.7 ± 1.8 ***
Oocyte size	15.7 ± 0.4	17.1 ± 0.4 *	17.8 ± 0.9 *
Granulosa cell layer size	13.2 ± 0.9	12.6 ± 1.2	17.0 ± 1.1 *
Follicular integrity			
Atresic follicles (%)	11.3 ± 2.4	22.4 ± 2.3 *	26.1 ± 3.7 **

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01; ***p>0.001. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 4. Effects of juvenile and peripubertal exposure to low doses of malathion on histological parameters of uterus

	Experimental groups		
Parameters	**Control (n=10 animals)**	**M10 (n=10 animals)**	**M50 (n=10 animals)**
Uterine morphometry (µm)			
Myometrium	164.7 ± 6.4	197.3 ± 8.2 **	168.6 ± 7.7
Endometrium	523.3 ± 26.7	505.7 ± 26.5	518.1 ± 32.4
Perimetrium	172.5 ± 9.9	174.0 ± 12.1	136.1 ± 9.8 *
Luminal epithelium	18.0 ± 0.5	23.8 ± 0.8 ****	22.8 ± 1.0 ****
Glandular epithelium	12.8 ± 0.5	12.2 ± 0.5	10.8 ± 0.4 ***
Number of glands	27.9 ± 1.6	25.5 ± 2.6	26.6 ± 5.4

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01; p<0.001; ****p>0.0001. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 5. Effects of juvenile and peripubertal exposure to low doses of malathion on oxidative profile of ovary

	Experimental groups		
Parameters	**Control (n=10 animals)**	**M10 (n=10 animals)**	**M50 (n=10 animals)**
Oxidative stress			
MDA concentration (nmol/mg protein)	1.7 ± 0.1	2.2 ± 0.1*	2.2 ± 0.20*
Antioxidants			
GSH (ug /mg protein)	26.1 ± 3.7	52.4 ± 2.6	57.0 ± 4.7
Glutathione transferase (GST, mM/mg protein)	18.0 ± 2.1	8.4 ± 2.4*	7.9 ± 1.2*
Catalase (Cat mM / mg protein)	8.1 ± 2.3	7.1 ± 2.8	6.9 ± 2.9
Superoxide dismutase activity (SOD U mg protein^{-1})	0.02 ± 0.005	0.01 ± 0.003	0.003 ± 0.001*

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

Table 6. Effects of juvenile and peripubertal exposure to low doses of malathion on oxidative profile of uterus

	Experimental groups		
Parameters	**Control (n=10 animals)**	**M10 (n=10 animals)**	**M50 (n=10 animals)**
Oxidative stress			
MDA concentration (nmol/mg protein)	2.4 ± 0.1	2.0 ± 0.1	1.8 ± 0.1*
Antioxidants			
GSH (ug /mg protein)	31.5 ± 2.1	27.6 ± 2.4	26.8 ± 0.7
Glutathione transferase (GST, mM/mg protein)	6.0 ± 0.3	12.9 ± 3.5*	25.1 ± 11.3*
Catalase (Cat mM / mg protein)	18.6 ± 4.2	26.2 ± 4.6	21.25 ± 3.1
Superoxide dismutase activity (SOD U mg protein^{-1})	0.01 ± 0.003	0.005 ± 0.001*	0.005 ± 0.001*

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. **p<0.05. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

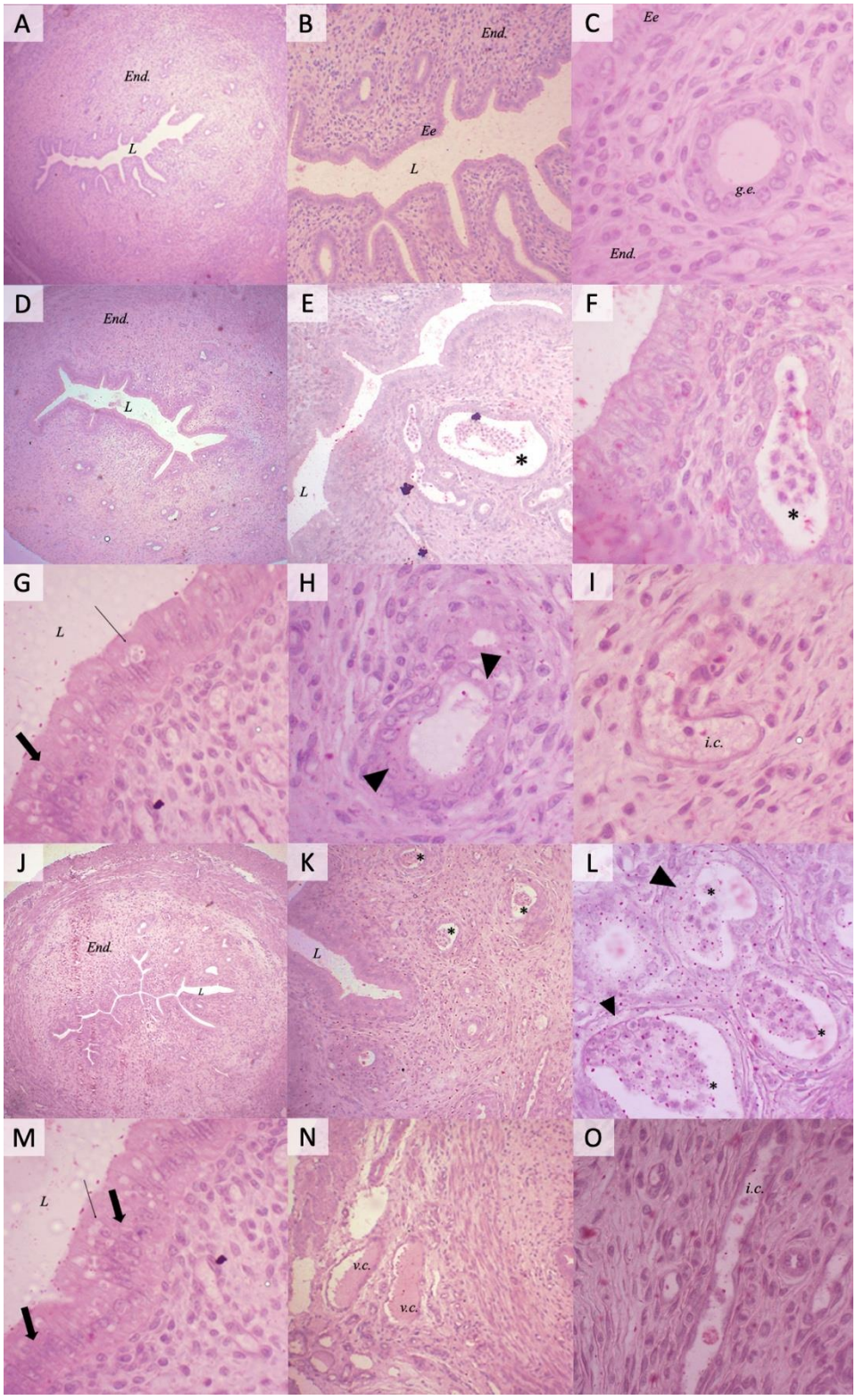

Figure 1. Effects of juvenile and peripubertal exposure to low doses of malathion on uterus histopathology Photomicrographs of uterus sections from the control (A-C), M10 (D-I) and M50 (J-O) of malathion groups. Control animals reveals normal appearance of luminal (A and B) and glandular (C) epithelium, and normal stroma of the uterine mucosa (A and B). (E,F,K and L) Desquamated cells in glandular lumen (asterisk). (G and M) Proliferative epithelium (thick arrow) and presence of vacuole in the epithelium (thin arrow). (H and L) Glandular epithelial polimorfism (arrowhead). (I, O) Inflammatory cells in (i.c.) and (N) vascular congestion (v.c.). M10 - rats treated with 10 mg kg−1 malathion; M50 - rats treated with 50 mg kg−1 malathion.End, endometrial stroma; l, lumen; ee, epithelial endometrium; ge, glandular epithelium. Haematoxylin and eosin stain (A,D,J: 40X magnification; B,E,K: 100X magnification; C, F-I, L-O: 400X magnification).

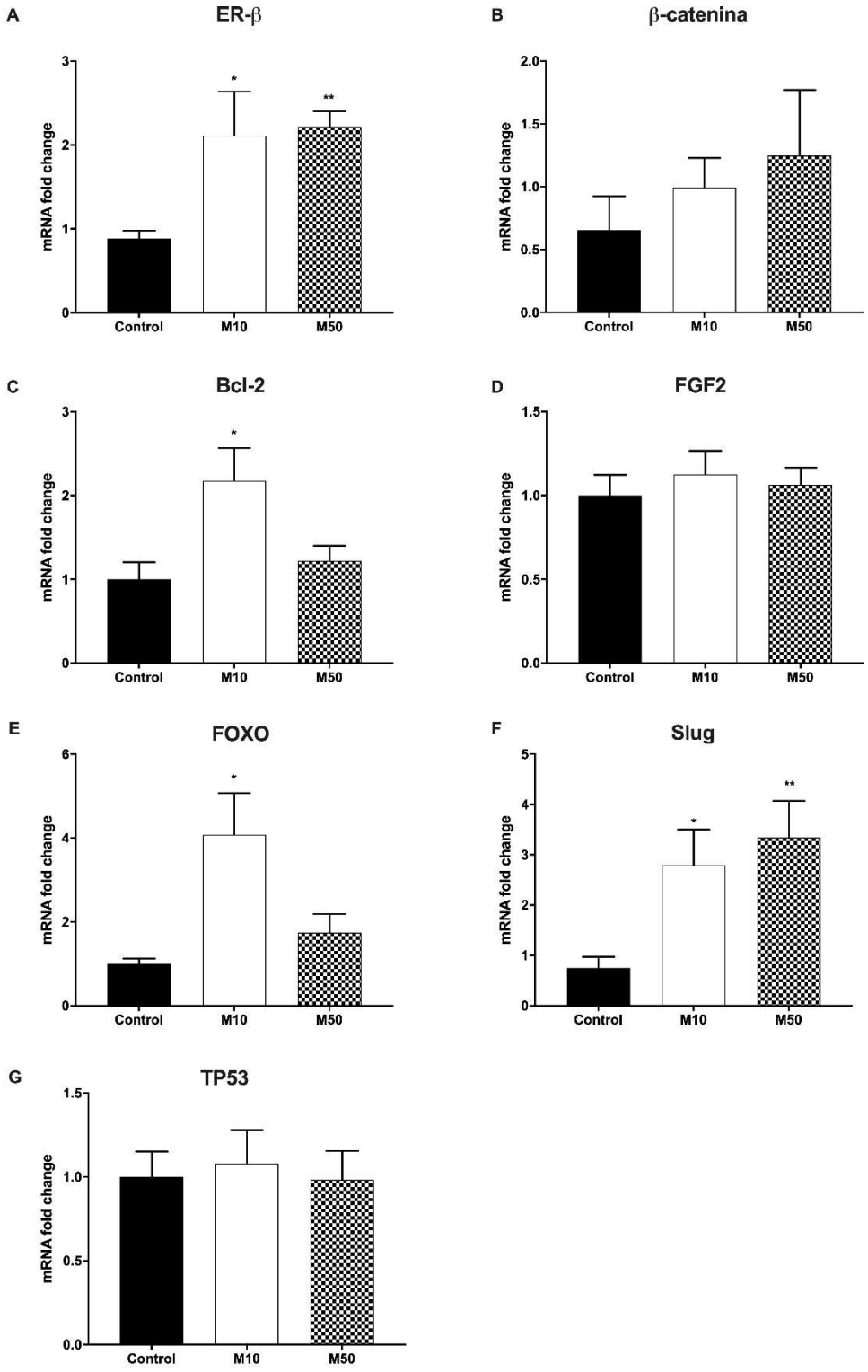

Figure 2. Effects of juvenile and peripubertal exposure to low doses of malathion on ovarian mRNA expression of (A) ER- β, (B) β-catenina, (C) Bcl-2, (D) FGF-2, (E) FOXO, (F) Slug and (G) TP53.
Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion.

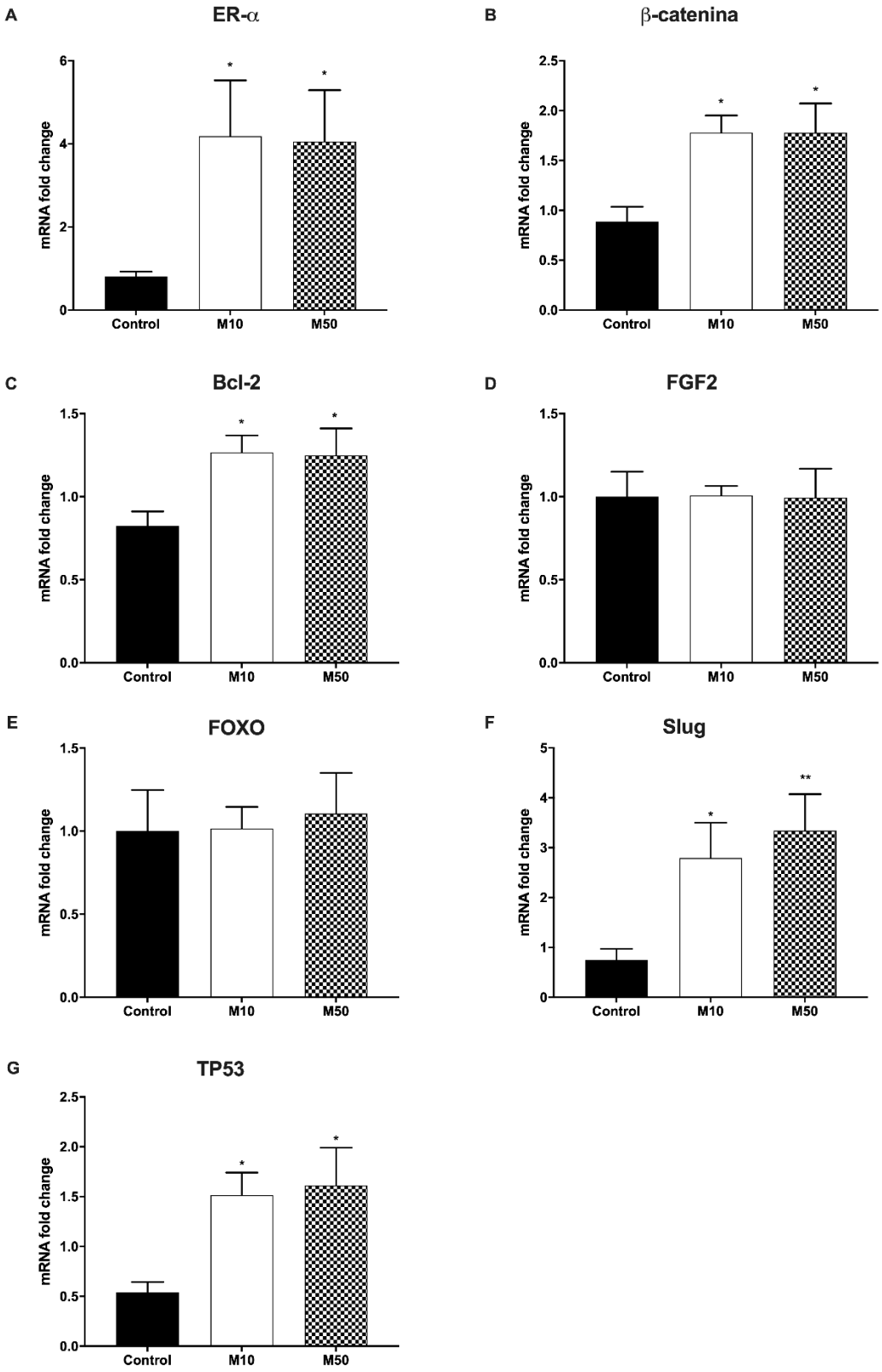

Figure 3. Effects of juvenile and peripubertal exposure to low doses of malathion on uterine mRNA expression of (A) ER-α, (B) β-catenina, (C) Bcl-2, (D) FGF-2, (E) FOXO, (F) Slug and (G) TP53.

Data are presented as the mean ± s.e.m. One-way ANOVA test with *a posteriori* Dunnett's test. *p<0.05; **p<0.01. M10 - rats treated with 10 mg kg-1 malathion; M50 - rats treated with 50 mg kg-1 malathion

8 CONSIDERAÇÕES FINAIS

A contribuição do presente estudo se deu pela avaliação dos efeitos da exposição a doses consideradas baixas de malathion sobre o desenvolvimento dos sistemas reprodutor feminino e masculino, bem como pela verificação de mecanismos oxidativos e de expressão gênica envolvidos em possíveis danos. As doses abordadas foram consideradas baixas quando comparadas a estudos anteriores, à toxicidade sistêmicas e NOAEL para desenvolvimento reprodutivo de ratos.

Em relação ao sistema reprodutor masculino, a novidade para a literatura foi de que a exposição de ratos a baixas doses de malathion durante os períodos juvenil e peripuberal prejudicou a integridade acrossômica de espermatozoides através da indução de estresse oxidativo no gameta masculino e alteração na expressão de genes envolvidas na biossíntese de testosterona. Concluímos ainda que, as células de Leydig são sensíveis ao dano oxidativo e sua função de síntese de andrógeno foi prejudicada especialmente após a exposição a menores concentrações de malathion.

Já em relação aos efeitos do inseticida sobre parâmetros reprodutivos femininos, foi observado que a exposição de folículos pré-antrais *in vitro* ao malation prejudicou a integridade e desenvolvimento folicular através da diminuição de níveis de estradiol, mas independente de estresse oxidativo. Já a exposição de ratas a baixas doses de malathion durante os períodos juvenil e peripuberal prejudicou tanto a integridade de folículos ovarianos quanto do endométrio uterino independente de alterações nos níveis hormonais. Os mecanismos envolvidos nesses danos abrangem a alteração do perfil oxidativo tanto em ovários quanto em útero, e a alteração da expressão de genes relacionados com a regulação do ciclo celular, inibição da apoptose e ação da via endócrina.

Ressaltamos ainda que não existiam estudos na literatura que avaliaram

mecanismos envolvidos nos danos causados a parâmetros reprodutivos após exposição a baixas doses de malathion, especialmente durante os períodos juvenil e peripuberal. Também não havia um único estudo que avaliou tanto parâmetros de integridade e desenvolvimento folicular quanto perfil oxidativo de folículos ovarianos expostos ao malathion em modelo *in vitro*.

A partir dos estudos supracitados, concluímos que o malathion pode prejudicar a integridade de folículos e o desenvolvimento pós-natal dos sistemas reprodutor feminino e masculino através da alteração de perfil oxidativo e expressão de genes envolvidos nas vias de sinalização endócrina e de controle de proliferação celular.

Por fim, o objetivo central é conscientizar o uso do malation e buscar minimizá-lo tanto quanto possível dados os efeitos adversos do composto ao sistema reprodutor e prejuízo à fertilidade masculina e feminina mesmo em baixas doses. Políticas governamentais podem ser melhor incentivadas e aplicadas como métodos alternativos de controle para as arboviroses.

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ANEXOS

ANEXO A
Aprovação de Projeto pelo Comitê de Ética (CEUA-UEL)

Projeto: Avaliação dos efeitos do inseticida Malation sobre o desenvolvimento do sistema genital masculino, gastrointestinal e renal de ratos desde o período juvenil até a peripuberdade

UNIVERSIDADE ESTADUAL DE LONDRINA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 137/2016 **Londrina, 13 de Julho de 2016.**

Prezada Pesquisadora,

Certificamos que o projeto intitulado **"Avaliação dos efeitos do inseticida Malation sobre o desenvolvimento do sistema genital masculino, gastrointestinal e renal de ratos desde o período juvenil até a puberdade"**, protocolo CEUA n° **12305.2016.65**, sob a responsabilidade de **Glaura Scantamburlo Alves Fernandes**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **05/07/2016**.

O objetivo do projeto é avaliar se a exposição ao Malation durante o período peripuberal poderá trazer prejuízos para o desenvolvimento dos sistemas genital masculino, gastrointestinal, renal e hepático de ratos. Os animais serão distribuídos casualmente em três grupos experimentais (n=25 animais/grupo). Dois grupos de animais serão tratados com Malation nas doses de 10 mg/Kg ou 50 mg/Kg de peso corpóreo via gavage. Essas doses correspondem a 0,5% e 2,5%, respectivamente, da DL50 oral para ratos (DL50 oral =2000 mg/kg) (U S EPA, 2000). O outro grupo (grupo controle) receberá apenas o veículo (óleo de soja) em igual volume. No 80º dia experimental os ratos serão anestesiados com a associação de xilazina e quetamina e mortos por punção cardíaca para a coleta do sangue em tubo heparinizado (hepararina sódica) para dosagens hormonais. GI 1

Vigência do Projeto	30/08/2016 a 30/08/2019
Espécie/linhagem	Rato heterogênico / Wistar
Nº de animais	75
Peso/Idade	Indeterminado / 22 dias
Sexo	Machos
Origem	Biotério Central / UEL
Amostras a serem coletadas	Testículos, epidídimos, vesícula seminal, próstata, sangue, fígado, estômago, rim, intestino, fêmur

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente.

Prof. Dr. João Waine Pinheiro
Vice-Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora do Projeto
Departamento de Biologia Geral / Centro de Ciências Biológicas
Com cópia Waldiceu A. Verri Junior (Coord. do Biotério Central/UEL); Chefe do Departamento de Biologia Geral e Diretor(a) do Centro de Ciências Biológicas

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 10.011 - CEP 86057-970 - Internet http://www.uel.br
LONDRINA - PARANÁ - BRASIL

ANEXO B

Aprovação de Projeto pelo Comitê de Ética (CEUA-UEL)
Projeto: Avaliação dos efeitos do inseticida Malation sobre o desenvolvimento do sistema genital feminino de ratos desde o período juvenil até a puberdade

UNIVERSIDADE ESTADUAL DE LONDRINA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 01/2020

Londrina, 22 de Janeiro de 2020.

Prezado (a) professor (a),

Certificamos a aprovação do projeto de pesquisa intitulado: "**Avaliação dos efeitos do inseticida malation sobre o desenvolvimento do sistema genital feminino de ratos desde o período juvenil até a puberdade,**" protocolo CEUA n° **21053.2019.71** sob a responsabilidade de **Glaura Scantamburlo Alves Fernandes**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) em **22/01/2020**.

Este projeto tem por objetivo avaliar se a exposição ao malation durante o período peripuberal poderá trazer prejuízos sobre a morfofisiologia de útero e ovários de ratas Wistar. **Grau de invasividade: 2.**

Finalidade	( ) Ensino (x) Pesquisa científica
Vigência da autorização	01/04/2020 a 01/04/2023
Espécie/ linhagem/ raça	Rato heterogênico/ Wistar
N° de animais	30
Peso/ Idade	21 dias
Sexo	Fêmeas
Origem	Biotério Central da UEL/ CCB
Amostras a serem coletadas	Sangue, útero e ovários

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Em cumprimento às exigências do CONCEA, em até 30 dias da finalização do projeto de pesquisa ou extensão, conforme vigência expressa neste ofício, encaminhar relatório da descrição de uso de animais para ceua@uel.br, conforme modelo disponível no site da CEUA/UEL (http://www.uel.br/comites/ceua/pages/relatorio-de-projetos.php).

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente.

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Londrina
ceua@uel.br / (43) 3371-5454

Ilmo.(a) Sr.(a)
Prof. (a) Dr (a). Glaura Scantamburlo Alves Fernandes
Responsável pelo projeto
C/C para Depto. de Biologia Geral /CCB
C/C para a Direção do Centro de Ciências Biológicas/CCB
C/C para o Biotério Central do CCB

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 10.011 - CEP 86057-970 – Internet http://www.uel.br
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ANEXO C

Guide for Authors

Journal of Comparative Pathology:

https://www.elsevier.com/journals/journal-of-comparative-pathology/0021-9975/guide-for-authors

Toxicology in vitro

https://www.elsevier.com/journals/toxicology-in-vitro/0887-2333/guide-for-authors

Journal of Developmental Origins of Health and Disease:

https://www.cambridge.org/core/journals/journal-of-developmental-origins-of-health-and-disease/information/instructions-contributors

Toxicology Letters:

https://www.elsevier.com/journals/toxicology-letters/0378-4274/guide-for-authors

APÊNDICES

APÊNDICE A

Considerações finais