PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIAS DA SAÚDE

MANOEL CARLOS MELILLO FELZENER

CARACTERIZAÇÃO MORFOLÓGICA DO ÚTERO DE RATAS ADULTAS EXPOSTAS DURANTE O PERÍODO PERINATAL A UMA MISTURA DE DESREGULADORES ENDÓCRINOS

Presidente Prudente – SP
2022

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIAS DA SAÚDE

MANOEL CARLOS MELILLO FELZENER

CARACTERIZAÇÃO MORFOLÓGICA DO ÚTERO DE RATAS ADULTAS EXPOSTAS DURANTE O PERÍODO PERINATAL A UMA MISTURA DE DESREGULADORES ENDÓCRINOS

Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para Obtenção do título de Mestre – Área de Concentração: Ciências da Saúde

Orientador:
Prof. Dr. Leonardo de Oliveira Mendes

Presidente Prudente – SP
2022

Catalogação Internacional na Publicação (CIP)

636.808
F329c

Felzener, Manoel Carlos Melillo

Caracterização morfológica do útero de ratas adultas expostas durante o período perinatal a uma mistura de desreguladores endócrinos \ Manoel Carlos Melillo Felzener; orientador Leonardo de Oliveira Mendes.– Presidente Prudente, 2022.

51 f. : il.

Dissertação (Mestrado em Ciências da Saúde) – Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2022.

Bibliografia.

1. Desreguladores endócrinos. 2. Histoarquitetura. 3. Exposição ambiental. 4. Útero. 5. DOHaD. I. Mendes, Leonardo de Oliveira, orient. II. Título.

Bibliotecária: Jakeline Margaret de Queiroz Ortega - CRB 8/6246

MANOEL CARLOS MELILLO FELZENER

CARACTERIZAÇÃO MORFOLÓGICA DO ÚTERO DE RATAS ADULTAS EXPOSTAS DURANTE O PERÍODO PERINATAL A UMA MISTURA DE DESREGULADORES ENDÓCRINOS

Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre EM Ciências da Saúde – Área de Concentração: Ciências da Saúde.

Presidente Prudente, 28 de outubro de 2022

BANCA EXAMINADORA

_____

Prof. Dr. Leonardo de Oliveira Mendes
Universidade do Oeste Paulista – Unoeste
Presidente Prudente - SP

_____

Prof. Dr. Renata Calciolari Rossi
Universidade do Oeste Paulista – Unoeste
Presidente Prudente - SP

_____

Dra. Patricia Villela e Silva
Instituto de Biociências de Botucatu – IBB/UNESP
Botucatu - SP

DEDICATÓRIA

À Deus, pois sem Ele nada seria possível.

Aos meus pais, Manoel e Zoe (*in memorian*), por sempre acreditarem em mim.

À minha amada esposa Lorraine e meus filhos Pedro e Manuela, minha razão de viver, por todo amor, incentivo, apoio e compreensão. Nada disso teria sentido se vocês não existissem na minha vida.

AGRADECIMENTOS

Agradeço ao meu orientador, Prof. Dr. Leonardo de Oliveira Mendes, por me receber como aluno do mestrado. Obrigado pela confiança no meu trabalho, pelo respeito, por me ensinar, pela compreensão, pelos sábios conselhos e por me dar todo o suporte que necessitei ao longo desses anos.

À Prof. Dra. Renata Calciolari Rossi e Profa. Dra. Patricia Vilella e Silva, por sua boa vontade e disponibilidade em poder estar presente na minha defesa, me dando todo o suporte, críticas e sugestões para que esse trabalho fosse realizado da melhor forma e que tivesse contribuição relevante com a sociedade.

Muito obrigado aos membros do Programa de Pós-graduação em Ciências da Saúde da UNOESTE de Presidente Prudente/SP, em especial aos professores do programa. Seus ensinamentos foram muitos e certamente contribuíram positivamente para a minha formação.

Aos responsáveis pelo Biotério da UNOESTE pelo trato tão zeloso com os animais utilizados e por disporem de seu tempo para que esse trabalho pudesse se concretizar. Muito obrigado.

Agradeço também a todos os colegas do Programa de Pós Graduação em Ciências da Saúde de Presidente Prudente/SP, pessoas com as quais tive o prazer de conviver, infelizmente por pouco tempo, mas que tornaram tudo mais fácil.

Por fim, agradeço à minha amada família, meus pais (*in memorian*) Manoel de Araújo Felzener e Zoe Terezinha Melillo Felzener, à minha esposa Lorraine Reis de Carvalho Ferreira Felzener e meus filhos Pedro de Carvalho Ferreira Felzener e Manuela de Carvalho Ferreira Felzener. Vocês nunca mediram esforços para realizar meus sonhos e vontades. Tudo o que pedi a vocês, sempre fizeram o possível e o impossível para tornar real. Deram-me o seu melhor, vocês sempre estarão em meu pensamento e coração.

Contudo, a minha fé não me permite deixar de agradecer a Deus, pois “tudo posso naquele que me fortalece.”

"Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem timão nem bússola, nunca tendo certeza do seu destino". *(Leonardo da Vinci)*

RESUMO

Caracterização morfológica do útero de ratas adultas expostas durante o período perinatal a uma mistura de desreguladores endócrinos

Desreguladores endócrinos (DE) são compostos exógenos dispersos no ambiente e que modificam a dinâmica hormonal, causando diversas consequências para a saúde humana, incluindo doenças crônicas, como o câncer. A maioria dos estudos é referente à avaliação dos efeitos de DE isolados ou em pequenos grupos e, assim, não mimetizam a exposição total à qual os seres humanos estão submetidos. Desta forma, pesquisas que mimetizam a exposição humana durante o período perinatal, apoiado na proposta de DOHaD, são importantes para caracterizar os efeitos dos DE na saúde/homeostase a longo prazo, principalmente em órgãos hormônio-dependentes, como o útero. Fêmeas prenhas da linhagem Sprague-Dawley foram divididas em 2 grupos experimentais (Ctrl [veículo] e Mix DE diluídos em óleo de milho, por gavagem) e expostas durante a gestação e lactação a uma mistura de 12 compostos sintéticos incluindo ftalatos, agroquímicos, filtros U.V., bisfenol A e butilparabeno. Após a lactação, os filhotes fêmeas da geração F1 foram mantidas recebendo água e ração ad libtum até completarem 365 dias de idade, quando foram eutanasiadas e os dois cornos uterinos coletados. Fragmentos uterinos foram processados e lâminas histológicas confeccionadas, sendo submetidas às análises histopatológicas, estereológicas e morfométricas. Nos animais submetidos à mistura de DE observou-se redução da espessura do compartimento endometrial e aumento das camadas musculares interna e externa. No epitélio uterino dos animais expostos ao DE observou-se aumento da altura e alteração do fenótipo nuclear, porém sem alteração na dimensão fractal. Por outro lado, a mistura de DE não influenciou a produção de mucinas e o número de mastócitos, bem como no peso dos órgãos. Assim, podemos concluir que a exposição perinatal a uma mistura de DE que mimetiza a exposição humana altera a morfologia uterina, o que pode gerar impactos negativos em processos reprodutivos.

Palavras-chave: Desreguladores endócrinos; Histoarquitetura; Exposição ambiental; Útero; DOHaD

ABSTRACT

Morphological characterization of the uterus of adult rats exposed during the perinatal period to a mixture of endocrine disregulators

Endocrine disruptors (ED) are exogenous compounds dispersed in the environment that modify hormonal dynamics, causing several consequences for human health, including chronic diseases such as cancer. Most studies refer to the evaluation of the effects of ED alone or in small groups and, therefore, do not mimic the total exposure to which human beings are subjected. Thus, studies that mimic human exposure during the perinatal period, supported by the DOHaD proposal, are important to characterize the effects of ND on long-term health/homeostasis, especially in hormone-dependent organs, such as the uterus. Pregnant Sprague-Dawley females were divided into 2 experimental groups (Ctrl [vehicle] and Mix DE diluted in corn oil, by gavage) and exposed during pregnancy and lactation to a mixture of 12 synthetic compounds including phthalates, agrochemicals, filters U.V., bisphenol A and butylparaben. After lactation, the female offspring of the F1 generation were kept receiving water and food ad libtum until they were 365 days old, when they were euthanized and the two uterine horns were collected. Uterine fragments were processed and histological slides were prepared and submitted to histopathological, stereological and morphometric analyses. In the animals submitted to the DE mixture, a reduction in the thickness of the endometrial compartment and an increase in the inner and outer muscle layers was observed. In the uterine epithelium of the animals exposed to DE, an increase in height and alteration of the nuclear phenotype were observed, but without alteration in the fractal dimension. On the other hand, the DE mixture did not influence the production of mucins and the number of mast cells, as well as the weight of the organs. Thus, we can conclude that perinatal exposure to an ED mixture that mimics human exposure alters uterine morphology, which can negatively impact reproductive processes.

Keywords: Endocrine disruptors; Histoarchitecture; Environmental exposure; Uterus; DOHaD

LISTA DE SIGLAS

BPA - Bisfenol A

Ctrl - Grupo controle (óleo de milho)

DEHP - di-(2-etilhexil) ftalato

DBP - di-n- butil ftalato

DDT - diclorodifenil-dicloroetileno

DOHaD - Developmental Origins of Health and Disease

DG - Dia gestacional

DPN - Dia pós natal

DE - Desregulador endócrino

DF - Dimensão fractal

EPA - Environmental Protection Agency

ED Mix - Grupo experimental (óleo de milho + mistura de desreguladores)

HE - Hematoxilina e Eosina

ME - Miométrio Externo

MI - Miométrio Interno

OMC - metoxinamato de octila

SD - *Sprague-Dawley*

4-MBC - 4-metil-benzidileno cânfora

LISTA DE TABELAS

LISTA DE FIGURAS

SUMÁRIO

CARACTERIZAÇÃO MORFOLÓGICA DO ÚTERO DE RATAS ADULTAS EXPOSTAS DURANTE O PERÍODO PERINATAL A UMA MISTURA DE DESREGULADORES ENDÓCRINOS

Manoel Carlos Melillo Felzener[1], Thainá Cavalleri Sousa[2], Odin Serodio Mettifogo[3], Maria Luiza Silva Ricardo[2], Karianne Delalibera Hinokuma[2], Leonardo de Oliveira Mendes[1,2]

[1]Mestrado em Ciências da Saúde - Universidade do Oeste Paulista/UNOESTE, Presidente Prudente, SP, Brasil

[2]Programa de Pós-Graduação em Ciência Animal - Universidade do Oeste Paulista/UNOESTE, Presidente Prudente, SP, Brasil

[3]Faculdade de Medicina de Presidente Prudente - Universidade do Oeste Paulista/UNOESTE, Presidente Prudente, SP, Brasil

Autor correspondente:

Leonardo de Oliveira Mendes

Pró-Reitoria de Pesquisa e Pós-graduação Universidade do Oeste Paulista (UNOESTE)
Rodovia Raposo Tavares, km 572, Bairro do Limoeiro
CEP 19067-175
Presidente Prudente – SP

Email: leobio85@gmail.com

O trabalho está apresentado sob a forma de artigo, segundo as normas do periódico o qual será submetido: Environmental Science and Pollution Research, Fator de impacto: 5.190 Qualis: A

INTRODUÇÃO

Devido ao processo de industrialização, milhares de derivados químicos sintéticos foram desenvolvidos, porém nem todos passaram por avaliações de segurança rigorosas antes do uso comercial. Evidências científicas mostram que a população mundial foi afetada com o advento de novos desreguladores endócrinos (DE) presentes em diversos produtos de uso diário. Além disso, o aumento de doenças de caráter endócrino, aliada às malformações genitais e aos diversos tipos de tumores em órgãos hormônio-dependentes, como útero, próstata, ovário, testículo e mama, trouxeram grande visibilidade à área, tornando-a um interessante e atrativo campo de estudo para pesquisas experimentais e epidemiológicas (BERGMAN et al., 2013).

De acordo com a "Environmental Protection Agency" (EPA), um DE é definido como qualquer agente capaz de interferir na síntese, secreção, transporte, ligação ou eliminação de hormônios endógenos responsáveis pela manutenção da homeostase corpórea (KAVLOCK et al., 1996). Como revisado por Maqbool et al. (2016), cerca de 800 compostos químicos utilizados no cotidiano são capazes de desregular o ambiente hormonal e, dentre estes, somente uma pequena parte foi avaliada e teve seus mecanismos de ação elucidados. Apesar disso, um dos primeiros obstáculos nos estudos envolvendo tóxicos ambientais reside na fragmentação da exposição, onde os pesquisadores tendem a se concentrar na análise isolada ou em categorias específicas de desreguladores endócrinos encontrados no ar, solo, alimentos ou produtos do cotidiano, distanciando-se da exposição à qual os humanos estão submetidos. (RAPPAPORT; SMITH, 2010)

Sabe – se do potencial efeito negativo dos DE em uma diversidade de órgãos e sistemas, com especial apelo para aqueles que são hormônio-dependentes, visto que os DE causam perturbação direta na dinâmica de sensibilidade hormonal. Neste grupo destaca – se o útero, visto que para a regulação da homeostasia deste órgão a atividade de uma gama de hormônios é imprescindível (BERNARD et al., 2014; PARK et al., 2009; SEACHRIST et al., 2016).

Entre as classes de DE mais comumente utilizadas, encontra-se os ftalatos e bisfenol A (BPA), plastificantes usados para conferir flexibilidade aos produtos plásticos. Um dos ftalatos mais comuns, o di-(2-etilhexil) ftalato (DEHP), é liberado

dos produtos e causa efeitos tóxicos. O uso de DEHP foi contestado pelas autoridades européias devido às suas propriedades tóxicas. Com relação ao BPA, a maioria das pesquisas têm relacionado-o ao desenvolvimento do câncer de mama, (BINDER et al., 2018; SEACHRIST et al., 2016; SHAFEI et al., 2018) com estudos limitados quando se trata de outros tumores femininos, como câncer de ovário e colo do útero (MA et al., 2015; ORAL et al., 2016; PARK et al., 2009).

Com relação ao câncer endometrial, apenas um estudo de coorte foi relatado em humanos, relacionando a neoplasia com DE. Tal estudo mostrou que pacientes com hiperplasia endometrial pré-maligna e câncer de endométrio pós-menopausa tinham níveis séricos de BPA significativamente mais baixos do que os indivíduos saudáveis (HIROI et al., 2004), uma associação inesperada e que precisa de estudos futuros para confirmar. Em contraste, a exposição crônica de camundongos a baixas doses de BPA levou a uma proliferação epitelial aberrante no endométrio uterino(NEFF et al., 2019).

A maioria dos estudos envolvendo DE propôs uma exposição isolada ou a misturas constituídas de poucos DE da mesma classe. Ao invés de avaliar um a um ou grupos de dois a três fatores, o objetivo dessa abordagem é analisar simultaneamente o maior número destes (OLYMPIO et al., 2019). As evidências experimentais sobre os mecanismos através dos quais um conjunto mais amplo de DE se comporta em doses que se aproximam dos níveis ambientais ainda são escassos na literatura. Levando-se em conta este fato, Christiansen et al. (2012) estabeleceram uma mistura de 13 compostos químicos que foram selecionados como representativos de vários outros DE com o intuito de simular uma realística exposição humana (CHRISTIANSEN et al., 2012).

A composição da mistura, detalhadamente descrita por Christiansen et al. (2012), Axelstad et al. (2014), Isling et al. (2014), Boberg et al. (2015), constitui-se de 2 ftalatos: di-n- butil ftalato (DBP) e di-(2-etilexil) ftalato (DEHP); 5 agroquímicos: vinclozin, procloraz, procimidona, linuron, epoxiconazol; o metabólito do pesticida DDT, diclorodifenil-dicloroetileno (p,p‟-DDE). Esses 8 compostos são caracterizados por possuírem propriedades anti-androgênicas. Além disso, a mistura também contém quatro substâncias predominantemente estrogênicas: dois filtros u.v., o metoxinamato de octila (OMC) e o 4-metil-benzidileno cânfora (4-MBC); o composto fenólico bisfenol A (BPA); o preservativo antifúngico butilparabeno (AXELSTAD et al., 2014; BOBERG et al.,

2015; CHEON et al., 2002; ISLING et al., 2014).

No tocante ao risco futuro, quer seja de desenvolvimento neoplásico ou de qualquer outra condição patológica, apoiado no conceito de DOHaD (*Developmental Origins of Health and Disease*), sustenta – se a hipótese de que os eventos sofridos no início da vida (concepção, vida fetal e primeira infância) tem importância sumária no desenvolvimento de doenças futuras (HAGEMANN et al., 2021). Assim, voltamos nossa atenção aos efeitos causados à prole exposta na fase uterina e de lactação para que possamos compreender os impactos morfológicos futuros da exposição aos DE. Além disso, é bem estabelecido que diferentes tipos de efeitos podem ocorrer mesmo em baixas doses ambientalmente relevantes e, desta maneira, torna-se relevante o delineamento de estudos que avaliem os efeitos de misturas químicas no microambiente uterino.

MATERIAIS E MÉTODOS

Animais e Ambiente de Experimentação

24 fêmeas adultas (120 dias de idade, pesando aproximadamente 300g) e 12 machos adultos (90 dias de idade, pesando aproximadamente 300g) da linhagem *Sprague-Dawley (SD),* foram obtidos no Centro Multidisciplinar para Investigação Biológica na Área de Ciência de Animais de Laboratório (CEMIB/UNICAMP), e mantidos no Biotério de Pequenos Mamíferos da UNOESTE. Os animais foram distribuídos na proporção de duas fêmeas e um macho adulto para acasalamento por gaiola de polipropileno de 41x34x16cm, com tampa de aço inox na forma de grade e forradas com maravalha branca de pinho autoclavada, sendo as trocas das gaiolas e da maravalha realizadas 2 vezes por semana. Durante toda a fase de experimentação foram controladas as condições ambientais do biotério como: temperatura (média de 23°C a 25°C), umidade relativa do ar (55 ± 10%), período de luz (12 horas claro/12 horas escuro) e exaustão do ar contínua. Todos os animais receberam ração comercial livre de fitoestrógenos (NUVILABCR1/Nuvital-PR) e água filtrada em bebedouros de vidro com tampa e bico metálicos com capacidade para 500 mL. Água e ração foram fornecidas *ad libitum*. Os procedimentos de manuseio, administração de drogas, anestesia e eutanásia dos animais foram submetidos à Comissão de Ética no Uso de Animais da UNOESTE (Protocolo

CEUA 6034).

Mistura de DE

Os compostos DBP (Cas nº 175606-05-0), DEHP (Cas nº 117-81-7), Vinclozin (Cas nº 50-471-44-8), Procloraz (Cas nº 67747-09-5), Procimidona (Cas nº 32809-16- 8), Linuron (Cas nº 330-55-2), Epoxiconazol (Cas nº 133855-98-8), p,p-DDE (Cas nº 72- 55-9), 4-MBC (Cas nº 36861-47-9), OMC (Cas nº 5466-77-3), BPA (Cas nº 80-05- 07) e Butilparabeno (Cas nº 94-26- 8) foram adquiridos junto à Sigma-Aldrich (St Louis, Missouri, EUA). A mistura de DE foi diluída em óleo de milho (veículo) na concentração de 32,11 mg/kg/dia e administrada por via oral (gavagem) nos animais durante o período gestacional e lactacional.

Delineamento Experimental

A presença de esperma no esfregaço vaginal e a citologia vaginal positiva da fase de estro foram consideradas como indicadoras do dia gestacional 0 (DG0). As fêmeas consideradas prenhes foram mantidas individualmente nas gaiolas e divididas aleatoriamente em 2 grupos experimentais (n=10/grupo): Grupo Ctrl: (veículo: óleo de milho, por gavagem); Grupo ED Mix: 32,11 mg/kg/dia da mistura de DE diluídos em óleo de milho (2 ml/kg) por gavagem.

Os animais do grupo ED Mix receberam uma mistura de DE desenvolvida por Christiansen et al. (2012) e reproduzida por Axelstad et al. (2014), Isling et al. (2014), Boberg et al. (2015). A composição da mistura é descrita detalhadamente na Tabela 1, adaptada de Christiansen et al. (2012).

Tabela 1. Composição da mistura, consumo humano individual ajustado dos compostos químicos e misturas 100x

Compostos químicos	**Consumo humano ajustado e escolhido como base para estudo das misturas (mg/kg de peso corpóreo ao dia)**[1]	**Mistura 100x (mg/kg de peso corpóreo ao dia)**
DBP	0,01	1
DEHP	0,02	2
Vinclozin	0,009	0,9
Procloraz	0,014	1,4
Procimidona	0,015	1,5
Linuron	0,0006	0,06
Epoxiconazol	0,01	1
p,p‟-DDE	0,001	0,1
4-MBC	0,06	6
OMC	0,12	12
Bisfenol A	0,0015	0,15
Butilparabeno	0,06	6
Total (mg/kg)	0,32	32,11

[1]Ver Christiansen et al. (2012) para informações detalhadas sobre as estimativas do consumo humano e para as concentrações ajustadas que serão escolhidas como base para a mistura

A mistura de DE sofreu adaptação em relação à original, com remoção do paracetamol da sua composição, em virtude do potencial hepatotóxico que esta droga possui.

As ratas prenhes ou lactentes receberam o tratamento do dia gestacional 7 (DG7) até o dia pós-natal 21 (DPN21), sempre no mesmo período (8h – 10h). As fêmeas foram mantidas em gaiolas individuais e pesadas em dias alternados para permitir o cálculo do volume da mistura de DE a ser administrado e a investigação

de sinais clínicos de toxicidade.

Após o nascimento, o número de filhotes por ninhada foi reduzido para 8 (proporção entre machos e fêmeas de 1:1 sempre que possível), e ninhadas com número de filhotes inferior a sete não foram utilizadas na pesquisa.

No DPN 22 foi feito o desmame dos filhotes fêmeas, que ficaram alojadas em caixas contendo 2 animais cada. Os animais foram mantidos até atingirem 365 dias de idade, recebendo somente água e ração *ad libitum*, quando então foram eutanasiados por meio de aplicação intramuscular dos anestésicos xilazina e cetamina, seguido de exsanguinação.

Análise Biométrica

Após eutanásia, a remoção dos órgãos, os cornos uterinos, os ovários e glândula adrenal foram submetidos pesagem em balança eletrônica de precisão (Owa Labor, Oschatz, Alemanha) para obtenção dos dados biométricos.

Análise Morfométrica

Os fragmentos uterinos foram rapidamente fixados por imersão em metacarn (6 metanol: 3 clorofórmio: 1 ácido acético) e mantidos em álcool 70%. Posteriormente, o material foi desidratado em soluções crescentes de etanol, clarificadas em xilol e incluído em paraplast (Oxford Labware, St. Louis, MO, USA).

Os fragmentos incluídos em paraplast foram seccionados com 4µm de espessura em micrótomo rotativo e submetidos às seguintes colorações:

Hematoxilina - Eosina (HE): análise da dimensão fractal, análise morfométrica (altura epitelial, altura dos compartimentos e análise kariométrica).

Azul de Toluidina: quantificação de mastócitos.

Alcian Blue: quantificação de mucina

Análise Fractal

Para análise fractal foram analisadas secções histológicas de 8 animais/grupo, coradas com HE, sendo fotografadas (10 campos histológicos/secção, aumento de 40x), binarizadas para leitura e a dimensão fractal estimada pelo método box-counting, por meio do software Image J (Instituto

Nacional de Saúde, Estados Unidos – NIH), disponível gratuitamente na Internet (http://rsbweb.nih.gov/ij/). O software considerará o box-counting em duas dimensões, permitindo a quantificação da distribuição de pixels nesse espaço, não considerando, portanto, a textura da imagem. A influência disso é que duas imagens com a mesma distribuição dos pixels, uma binarizada e outra em níveis de cinza, possuirão a mesma dimensão fractal.

A análise das lâminas histológicas fractais se baseará na relação entre a resolução e a escala avaliada, e o resultado será quantitativamente expresso como a dimensão fractal do objeto que é DF= (Log Nr / log r-1), sendo Nr a quantidade de elementos iguais necessários para preencher o objeto original e r a escala aplicada ao objeto. Com isso, a dimensão fractal será calculada com o software Image J ficando sempre entre 0 e 2, não distinguindo texturas diferentes (FRANCHI et al., 2020).

Análise da Altura do Endométrio Uterino

A altura do epitélio uterino foi mensurada em secções histológicas coradas com H.E seguindo as instruções do software Image J (Instituto Nacional de Saúde, Estados Unidos – NIH), disponível gratuitamente na Internet (http://rsbweb.nih. gov/ij/). Foram analisadas secções histológicas de 08 animais/grupo, coradas com H.E, sendo fotografadas (10 campos histológicos/secção, aumento de 40x) e medidas 5 regiões distintas para cada imagem (AQUINO et al., 2019).

Análise da Altura dos Compartimentos Uterinos

A altura dos compartimentos uterinos (miométrio interno, miométrio externo e endométrio) foi mensurada em secções histológicas coradas com H.E seguindo as instruções do software Image J (Instituto Nacional de Saúde, Estados Unidos – NIH), disponível gratuitamente na Internet (http://rsbweb.nih. gov/ij/). Foram analisadas secções histológicas de 08 animais/grupo, coradas com H.E, sendo fotografadas (10 campos histológicos/secção, aumento de 40x) e medidas 5 regiões distintas para cada compartimento (RICHARDSON et al., 2018).

Análise Kariométrica

A análise kariométrica foi realizada nas células epiteliais do endométrio uterino de secções histológicas coradas com H.E de 08 animais/grupo, sendo fotografados 4 campos histológicos/secção, aumento de 100x e realizada a medição de 5 núcleos/secção aleatórios. Áreas de secção transversal nuclear (μm^2) e perímetros (μm) serão determinados para núcleos de 200 células secretoras epiteliais para obter o fator de forma [$=4\pi$.área nuclear/(nuclear perímetro)^2]. O fator de forma mede arredondamento e valores <1 estão associados a núcleos menos redondos (GONÇALVES et al., 2017).

Quantificação de Mucinas

Para a quantificação de mucina foram analisadas secções histológicas de 08 animais/grupo coradas com Alcian Blue, sendo fotografadas em aumento de 40x, 6 campos histológicos do lúmen uterino/secção. A área ocupada pela mucina (%) foi mensurada seguindo as instruções do software Image J (Instituto Nacional de Saúde, Estados Unidos – NIH), disponível gratuitamente na Internet (http://rsbweb.nih. gov/ij/). (MARTINEZ et al., 2010).

Quantificação de Mastócitos

Para a quantificação de mastócitos foram analisadas secções histológicas de 08 animais/grupo coradas com azul de toluidina, sendo fotografados, em aumento de 40x, todo o miométrio nestas secções, visto que este tipo celular encontra-se predominantemente neste compartimento. Os valores obtidos foram expressos em número de mastócitos/mm^2 (KARACA et al., 2008).

Análise estatística

Os valores obtidos após a realização das análises descritas acima foram submetidos ao teste de normalidade de Shapiro-Wilk. Os dados referentes ao ganho de peso corpóreo, peso relativo do ovário e adrenal, número de glândulas uterinas, análise fractal e quantificação de mastócitos desgranulados e totais apresentaram distribuição normal e foram submetidos ao Teste T. Os demais dados não passaram no teste de normalidade e, portanto, foram submetidos ao teste não paramétrico de

Mann-Whitney. Diferenças foram consideradas estatisticamente significantes quando $p<0,05$.

RESULTADOS

Com relação aos dados biométricos, não foram observadas diferenças em relação ao ganho de peso corpóreo, peso dos cornos uterinos, ovários e adrenal (Tabela 2).

Tabela 2. Ganho de peso corpóreo (g) e peso relativo (g/100g peso corpóreo) do útero, ovários e adrenal de ratas adultas expostas durante o período perinatal a uma mistura de DE baseada na exposição humana. Valores foram expressos mediana (Mínimo;Máximo) para útero e média±desvio padrão para os demais órgãos.

Orgão	Ctrl	ED Mix	Valor de P
Ganho de peso corpóreo	**236,4±14,67**	**253,6±7,59**	**0,285**
Útero	**0,246 (0,10;0,55)**	**0,242(0,16;0,53)**	**0,797**
Ovário Direito	**0,02±0,008**	**0,02±0,006**	**0,616**
Ovário Esquerdo	**0,02±0,005**	**0,02±0,007**	**0,773**
Adrenal	**0,01±0,004**	**0,01±0,001**	**0,106**

Para espessura dos compartimentos uterinos identificou – se redução do endométrio (Figura 1B e C) e aumento do miométrio tanto interno quanto externo no grupo ED Mix (Figura 1B, D e E). A redução da espessura do endométrio foi acompanhada da diminuição do número de glândulas uterinas neste compartimento (Figura 1B e F).

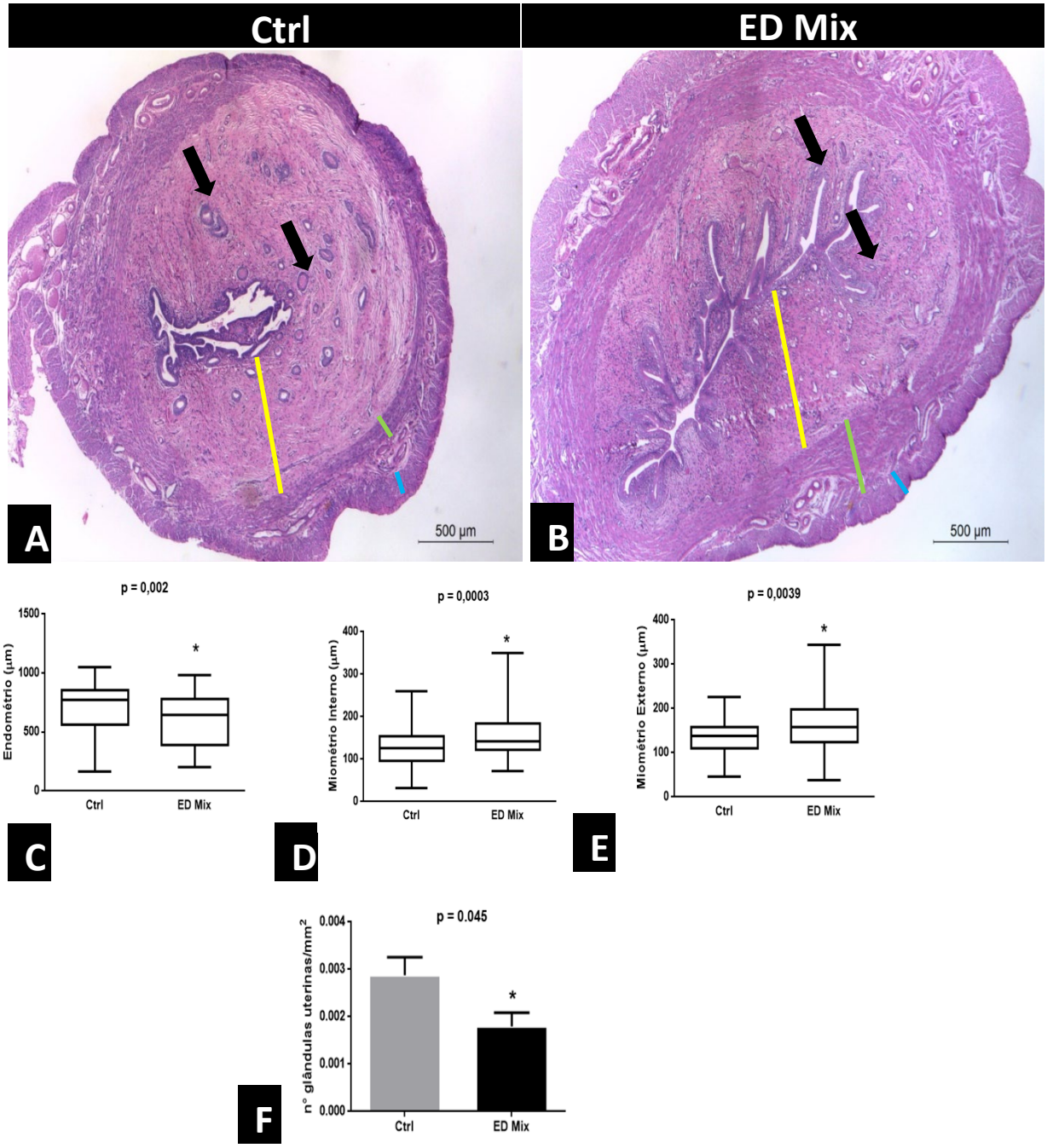


Figura 1. Secções histológicas do útero de ratas adultas do grupo Ctrl (A) e ED Mix (B) expostas durante o período perinatal a uma mistura de DE. Setas apontam as glândulas uterinas no compartimento endometrial. Linha amarela, verde e azul delimitam as camadas endometrial, muscular interna e externa, respectivamente. Análise morfométrica da espessura dos diferentes tipos de compartimento (C, D e E) e quantificação das glândulas uterinas (F). * $p \leq 0,05$. Coloração H&E.

Apesar da diminuição da espessura do compartimento uterino, foi observado aumento da altura epitelial após exposição à mistura de DE (Figura 2C e E). Além disso, o epitélio uterino dos animais do grupo ED Mix apresentou núcleos com fenótipo irregular e menos arredondados (Figura 2D, seta vermelha), evidenciado

pela redução do fator forma obtido na análise kariométrica (Figura 2G). Apesar das diferenças observadas pelas análises morfométricas, a análise fractal não apresentou diferenças entre os dois grupos experimentais (Figura 2F).

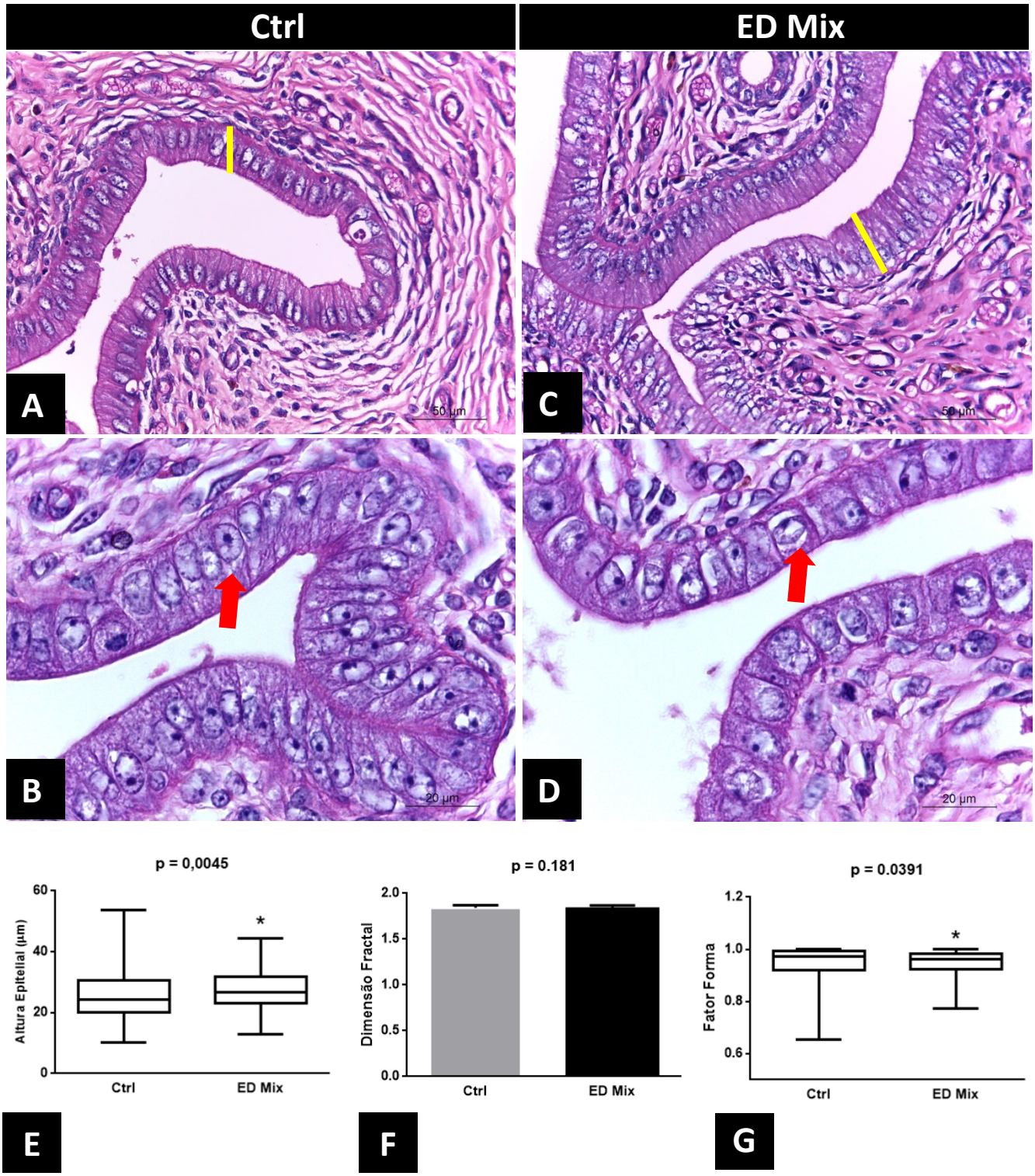


Figura 2. Secções histológicas do útero de ratas adultas do grupo Ctrl (A e B) e ED Mix (C e D) expostas durante o período perinatal à uma mistura de DE. Linhas amarelas representam a altura do epitélio e as setas vermelhas apontam os núcleos em ambos os grupos experimentais. Núcleos do grupo ED Mix (D) com formato irregular quando comparados com os núcleos do grupo Ctrl (C). Análise da altura epitelial (E), fractal (F) e kariométrica (G) nos dois grupos experimentais. * $p \leq 0,05$. Coloração H&E.

Ademais, verificou-se que a exposição aos DE não foi capaz de alterar a quantidade de mucina (Figura 3A-C) nem interferir na população de mastócitos, mostrando que a exposição à mistura de DE não desencadeou um processo inflamatório neste órgão (Figura 4A-E).

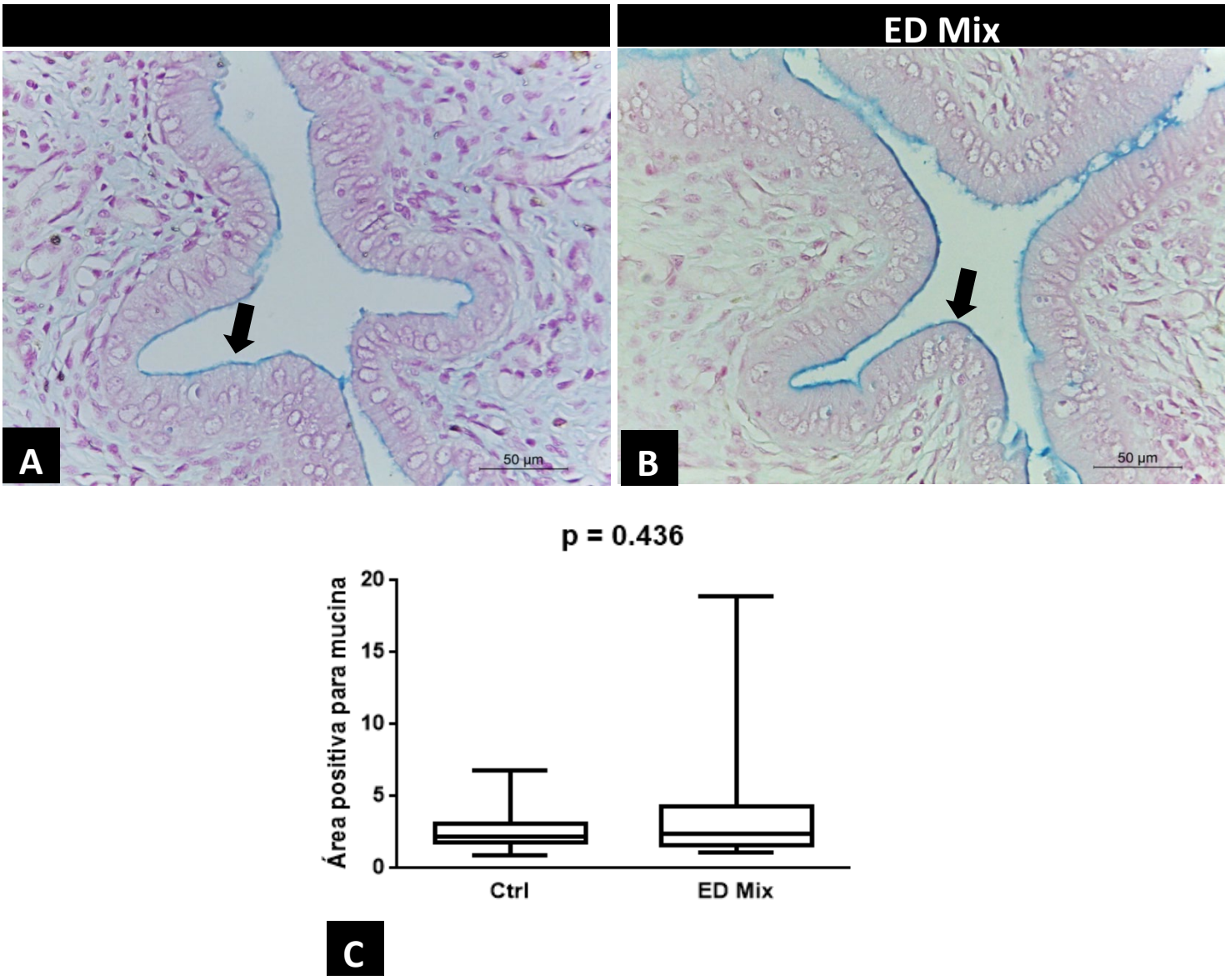


Figura 3. Secções histológicas do útero de ratas adultas do grupo Ctrl (A) e ED Mix (B) expostas durante o período perinatal a uma mistura de DE baseada na exposição humana. Setas pretas apontam a presença de mucina adjacente ao epitélio uterino. Quantificação de mucina (C) nos dois grupos experimentais. Coloração Alcian Blue.

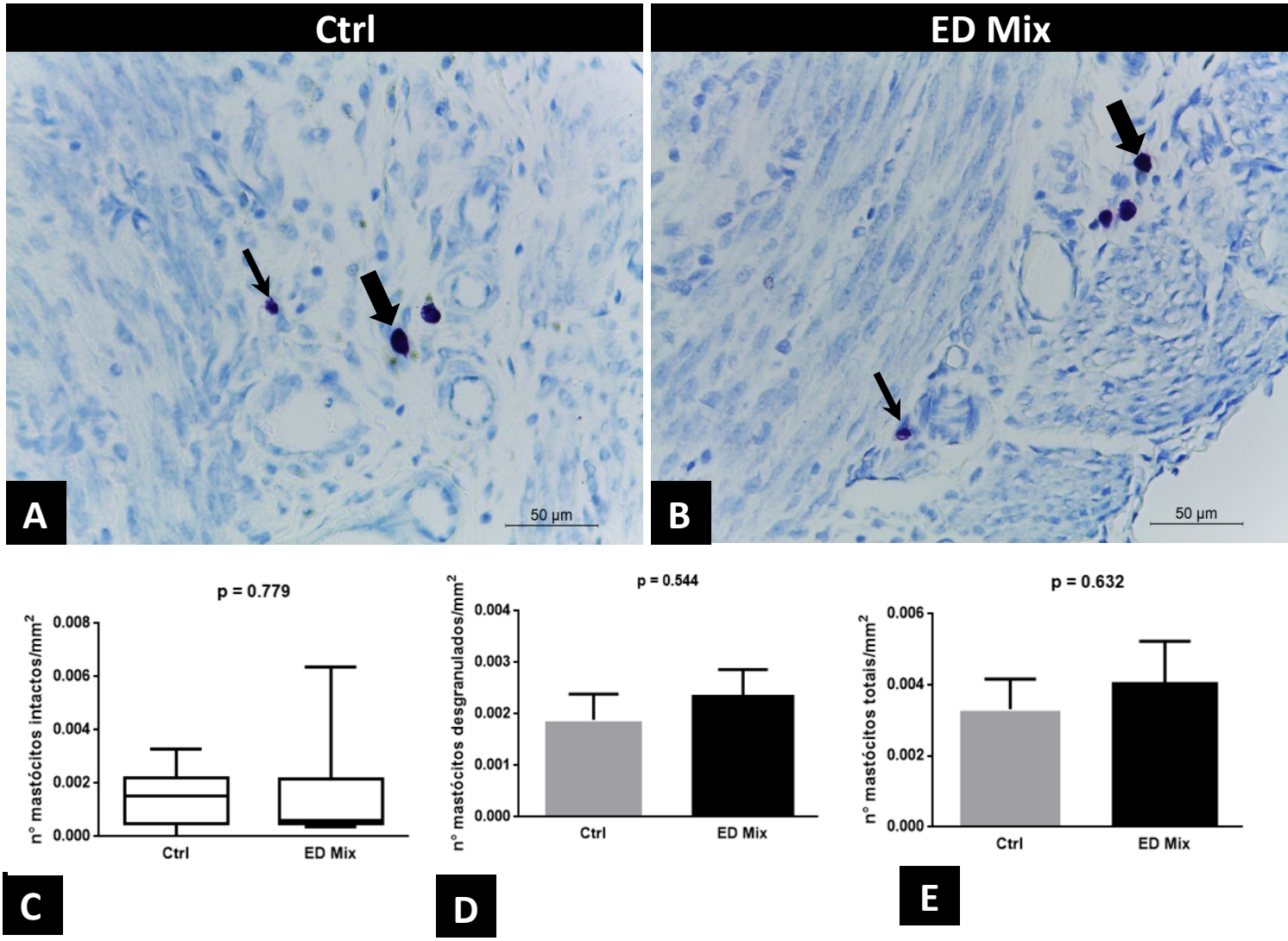


Figura 4. Secções histológicas do útero de ratas adultas do grupo Ctrl (A) e ED Mix (B) expostas durante o período perinatal à uma mistura de DE. Setas grossas apontam mastócitos intactos e setas finas indicam mastócitos desgranulados na camada muscular. Gráficos indicam o número de mastócitos intactos (C), desgranulados (D) e totais (E) nos dois grupos experimentais. Coloração Azul de Toluidina.

DISCUSSÃO

Os resultados apresentados no presente trabalho são inovadores ao comprovarem a hipótese inicial de que uma mistura de DE baseada na exposição humana desde a gestação até a fase adulta é capaz de alterar a histoarquitetura uterina, podendo comprometer as funções reprodutivas deste órgão.

Na literatura científica poucos são os trabalhos que dão enfoque à ação combinada de substâncias tóxicas e, quando isso acontece, apenas compostos de uma única classe são investigados, como o estudo realizado por Zhou e colaboradores, que observaram cistos ovarianos e redução dos índices de fertilidade em ratas expostas no período perinatal a uma mistura de ftalatos (ZHOU et al., 2017).

Cabe então salientar a relevância da utilização de uma mistura composta por múltiplas drogas, que agregue efeitos de diferentes classes de DE e, principalmente, permita sua interação orgânica no modelo experimental assim como ocorre com a exposição humana, visto que na dinâmica de vida estamos expostos simultaneamente e combinadamente a uma enorme gama de substâncias, sendo impossível isolar e avaliar efeitos individuais na exposição humana.

A exposição perinatal a uma mistura ambientalmente relevante de ftalatos, proposta no estudo de Zhou et al. (2017), induziu diminuição da distância anogenital na maior dose (500mg/kg de peso corpóreo) e aumento do peso uterino na menor dose (20µg/kg de peso corpóreo) (LI et al., 2020). Em nosso estudo não identificamos alterações de peso corpóreo e uterino, bem como do ovário e glândula adrenal. Vale ressaltar que o aumento do peso uterino relatado por Zhou et al. (2017) foi observado nas fêmeas no dia pós-natal (PDN) 8, não sendo constatada diferença aos 13 meses de idade. Isso ocorre pois no PDN8 os filhotes são sexualmente imaturos e dependentes da mãe. O eixo hipotálamo-hipófise-gonadal não está funcional no PDN 8, sendo os níveis hormonais da prole são dependentes da mãe, diretamente afetada pelos DE e, portanto, afetando o desenvolvimento pós-natal uterino e alterando o peso uterino de forma mais direta do que nos animais adultos (ZHOU et al., 2017). Em relação à manutenção do peso adrenal, sabendo de sua relação com a síntese de glicocorticóides, como o cortisol, sugere-se que não houve estresse crônico nestes animais capaz de induzir maior atividade e crescimento glandular.

A redução da espessura do endométrio observada em nosso estudo pode acarretar complicações na fertilidade, como destacado no trabalho de Zhou et al. (2017). Isto ocorre visto que a camada endometrial é a que desempenha o papel funcional mais pronunciado no processo reprodutivo e é responsável pela fixação e manutenção do embrião e uma redução em sua espessura implica diretamente em restrição à sua amplitude funcional (MARUYAMA & YOSHIMURA, 2008). Fenômeno semelhante foi observado por Zaid et al. (2021), que relatou diminuição da espessura do endométrio uterino em ratas adultas expostas ao bisfenol A durante seis semanas. O mesmo composto também já está relacionado com o aumento da espessura do miométrio de ratas adultas expostas ao DE durante 90 dias (OTHMAN et al., 2016). Os autores relacionam este fenótipo observado ao aumento da proliferação de células estromais, o que também foi descrito por Richardson et al. (2018), que identificou aumento do número de núcleos de células estromais PCNA-positivos após a administração oral de di-(2-ethylhexyl) phthalate (DEHP) em ratas adultas por 30 dias. O aumento da camada muscular pode estar relacionado com o desenvolvimento de leiomiomas uterinos, sendo os DE uma possível causa, visto que maiores concentrações de BPA foram encontradas neste tipo de tumor e em áreas adjacentes do miométrio (OTHMAN et al., 2016).

No que tange ao epitélio uterino, as análises demostraram um aumento da altura epitelial frente a exposição à mistura de DE. É bem reconhecido que tal alteração morfológica é uma resposta uterina à ação estrogênica (Padilla-Banks et al., 2001). Estudo avaliando a ação do glifosato demonstrou aumento da altura celular do epitélio uterino após 3 injeções subcutâneas do herbicida em ratas adultas (VARAYOUD et al., 2016). Tal resultado possui relevância clínica devido ao fato que estímulos no epitélio luminal uterino estão relacionados a desordens como endometriose e carcinoma endometrial (VAN LEEUWEN et al., 1994).

Os danos em nível celular mostraram – se presentes, uma vez que os animais do grupo ED Mix apresentaram alterações nucleares evidenciadas pela kariométrica. A arquitetura nuclear é um fator importante no funcionamento celular e na patogênese. Alterações neste parâmetro são reflexo de alterações genéticas e epigenéticas que podem estar relacionadas, inclusive, com o desenvolvimento de neoplasias (GONÇALVES et al., 2017). De fato, análises histopatológicas demonstram que os DE provocam alterações celulares significativas, como as descritas por Zaid et al. (2021). Os autores notaram, nos animais adultos expostos

via gavagem ao BPA durante 6 semanas, desorganização do epitélio uterino, com células cúbicas, apresentando núcleos irregulares núcleos irregulares e cromatina condensada. As células estromais do endométrio uterino também são responsivas à ação de DE, com desorganização tecidual deste compartimento além de hipertrofia celular em animais adultos expostos ao arsênio durante 4 semanas (AKRAM et al., 2010).

Consoante ao padrão sugestivo de redução de atividade, com menor endométrio e maiores camadas musculares, acompanhadas por núcleos menos globosos, é perceptível também a redução no número de glândulas no grupo ED Mix. Resultados discrepantes são encontrados na literatura científica envolvendo variação no número de glândulas uterinas e toxicantes ambientais. Enquanto Akram et al. (2010) relata que o arsênio reduz o número de glândulas uterina de maneira dose-dependente, Richardson et al. (2018) descreve o oposto, com aumento de glândulas no endométrio após exposição ao DEHP na dose mais alta de 200 mg/kg de peso corpóreo. A redução do número de glândulas, característica esta também observada em nosso estudo, pode ser consequência, segundo Akram et al. (2010), de uma perturbação do estroma endometrial. As glândulas endometriais sintetizam, secretam e transportam substâncias que são essenciais para o desenvolvimento do embrião e, por isso, qualquer neste padrão podem impactar na implantação e receptividade uterina (GRAY et al., 2001).

Na fase de desenvolvimento dos tecidos e órgãos, o organismo em desenvolvimento está mais suscetível às exposições ambientais. A exposição aos desreguladores endócrinos pode reprogramar o organismo em desenvolvimento, aumentando a susceptibilidade a doenças que se manifestam na vida adulta, processo denominado “reprogramação do desenvolvimento” ou “programação fetal” (WALKER, 2016). Alterações provocadas na fase de desenvolvimento embrionário apresentam desdobramentos futuros a longo prazo, como as descritas no presente estudo.

Salientamos que, embora para esse estudo as alterações estejam centradas na histoarquitetura do órgão e não tenhamos avaliado a implicação direta no poder reprodutivo, bem como no risco a carcinogênese, ainda não está claro se a exposição ao longo da vida pode aumentar o risco de câncer endometrial. Esta questão é de particular importância porque o crescimento e o desenvolvimento uterino continuam após o nascimento (BITENCOURT et al., 2019; BOSQUIAZZO et al., 2013;

COOKE et al., 2013)

CONCLUSÃO

Assim, podemos concluir que a exposição a uma mistura de DE durante o período perinatal promove alterações na histoarquitetura uterina de ratas adultas, podendo comprometer as funções do órgão, principalmente relacionadas aos processos reprodutivos.

REFERÊNCIAS

AKRAM, Z et al. Adverse effects of arsenic exposure on uterine function and structure in female rat. **Experimental Toxicology and Pathology**, v. 62, p. 451-459, 2010.

AQUINO, A. M. et al. Arsenic exposure during prepuberty alters prostate maturation in pubescent rats. **Reproductive Toxicology (Elmsford, N.Y.)**, v. 89, p. 136–144, out. 2019.

AXELSTAD, M. et al. Mixtures of endocrine-disrupting contaminants induce adverse developmental effects in preweaning rats. **Reproduction (Cambridge, England)**, v. 147, n. 4, p. 489–501, 2014.

BERGMAN, Å. et al. **State of the science of endocrine disrupting chemicals 2012**. [S.l.]: World Health Organization, 2013.

BERNARD, L. et al. Analytical methods for the determination of DEHP plasticizer alternatives present in medical devices: a review. **Talanta**, v. 129, p. 39–54, 2014.

BINDER, A. M. et al. Prepubertal and Pubertal Endocrine-Disrupting Chemical Exposure and Breast Density among Chilean Adolescents. **Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology**, v. 27, n. 12, p. 1491–1499, dez. 2018.

BITENCOURT, G. et al. Maternal exposure to triclosan causes fetal development restriction, deregulation of the oestrous cycle, and alters uterine tissue in rat offspring. **Environmental Toxicology**, v. 34, n. 10, p. 1105–1113, out. 2019.

BOBERG, J. et al. Perinatal exposure to mixtures of anti-androgenic chemicals causes proliferative lesions in rat prostate. **The Prostate**, v. 75, n. 2, p. 126–140, fev. 2015.

BOSQUIAZZO, V. L. et al. Perinatal exposure to diethylstilbestrol alters the functional differentiation of the adult rat uterus. **The Journal of Steroid Biochemistry and Molecular Biology**, v. 138, p. 1–9, nov. 2013.

BUCKLEY, J. P. et al. Prenatal exposure to environmental phenols and childhood fat mass in the Mount Sinai Children's Environmental Health Study. **Environment International**, v. 91, p. 350–356, maio 2016.

CHEON, Y.-P. et al. A genomic approach to identify novel progesterone receptor regulated pathways in the uterus during implantation. **Molecular Endocrinology (Baltimore, Md.)**, v. 16, n. 12, p. 2853–2871, dez. 2002.

CHRISTIANSEN, S. et al. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. **International Journal of Andrology**, v. 35, n. 3, p. 303–316, jun. 2012.

COOKE, P. S. et al. Uterine glands: development, function and experimental model

systems. **Molecular Human Reproduction**, v. 19, n. 9, p. 547–558, set. 2013.

FRANCHI, F. F. et al. Fractal analysis and histomolecular phenotyping provides insights into extracellular matrix remodeling in the developing bovine fetal ovary. **Biochemical and Biophysical Research Communications**, v. 523, n. 4, p. 823–828, 19 mar. 2020.

GONÇALVES, B. F. et al. Dual action of high estradiol doses on MNU-induced prostate neoplasms in a rodent model with high serum testosterone: Protective effect and emergence of unstable epithelial microenvironment. **The Prostate**, v. 77, n. 9, p. 970–983, jun. 2017.

GRAY, C. A. et al. Developmental biology of uterine gland. **Bio Reprod**, v. 65, p. 1311-1323, 2001.

HAGEMANN, E. et al. Developmental Origins of Health and Disease (DOHaD): The importance of life-course and transgenerational approaches. **Paediatric Respiratory Reviews**, v. 40, p. 3–9, dez. 2021.

HIROI, H. et al. Differences in serum bisphenol a concentrations in premenopausal normal women and women with endometrial hyperplasia. **Endocrine Journal**, v. 51, n. 6, p. 595–600, dez. 2004.

ISLING, L. K. et al. Late-life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters. **Reproduction (Cambridge, England)**, v. 147, n. 4, p. 465–476, 2014.

KARACA, T. et al. Distribution and heterogeneity of mast cells in female reproductive tract and ovary on different days of the oestrus cycle in Angora goats. **Reproduction in Domestic Animals = Zuchthygiene**, v. 43, n. 4, p. 451–456, ago. 2008.

KALSOOM, B. S. et al. Adverse effects of arsenic exposure on uterine function and structure in female rat. **Exp Toxicol Pathol**, v.62, p.451-459, jul. 2010.

KAVLOCK, R. J. et al. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. **Environmental health perspectives**, v. 104, n. suppl 4, p. 715–740, 1996.

LI, K. et al. Prenatal exposure to a phthalate mixture leads to multigenerational and transgenerational effects on uterine morphology and function in mice. **Reproductive Toxicology (Elmsford, N.Y.)**, v. 93, p. 178–190, abr. 2020.

MA, X.-F. et al. IKKβ/NF-κB mediated the low doses of bisphenol A induced migration of cervical cancer cells. **Archives of Biochemistry and Biophysics**, v. 573, p. 52–58, 2015.

MAQBOOL, F.; Mostafalou S, Bahadar H, et al. Review of endocrine disorders associated with environmental toxicants and possible involved mechanisms. Life Sci, v. 145, p. 265-73, 2016.

MARTINEZ, C. A. R. et al. Quantification by computerized morphometry of tissue levels of sulfomucins and sialomucins in diversion colitis in rats. **Acta Cirúrgica Brasileira**, v. 25, p. 231–240, jun. 2010.

MARUYAMA, T.; YOSHIMURA, Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. **Endocr J**, v. 55, p. 795-810. Jun. 2008.

MORONI, J. L.; DYCK, M. Desenvolvimento embrionário: quando avaliar o fenótipo e sua relação com o genótipo. **Revista Brasileira de Reprodução Animal**, v. 45, n. 4, p. 302–308, 2021.

NEFF, A. M. et al. Chronic Exposure of Mice to Bisphenol-A Alters Uterine Fibroblast Growth Factor Signaling and Leads to Aberrant Epithelial Proliferation. **Endocrinology**, v. 160, n. 5, p. 1234–1246, 1 maio 2019.

OLYMPIO, K. P. K. et al. O expossoma humano desvendando o impacto do ambiente sobre a saúde: promessa ou realidade? **Revista de Saúde Pública**, v. 53, 31 jan. 2019.

ORAL, D. et al. Epithelial-Mesenchymal Transition: A Special Focus on Phthalates and Bisphenol A. **Journal of Environmental Pathology, Toxicology and Oncology: Official Organ of the International Society for Environmental Toxicology and Cancer**, v. 35, n. 1, p. 43–58, 2016.

OTHMAN, E. R. et al. Bisphenol A Concentrates Preferentially in Human Uterine Leiomyoma and Induces Proliferation in Rat Myometrium. **Reprod Sci**, v. 23. p. 508 - 514, Oct. 2015.

PADILLA-BANKS, E. et al. The immature mouse is a suitable model for detection of estrogenicity in the uterotropic bioassay. **Environ Health Perspect**, v. 109, p. 821-826, Aug. 2001.

PARK, S.-H. et al. Cell growth of ovarian cancer cells is stimulated by xenoestrogens through an estrogen-dependent pathway, but their stimulation of cell growth appears not to be involved in the activation of the mitogen-activated protein kinases ERK-1 and p38. **The Journal of Reproduction and Development**, v. 55, n. 1, p. 23–29, fev. 2009.

RAPPAPORT, S. M.; SMITH, M. T. Environment and disease risks. **Science**, v. 330, n. 6003, p. 460–461, 2010.

RICHARDSON, K. A. et al. Di (2-ethylhexyl) phthalate (DEHP) alters proliferation and uterine gland numbers in the uteri of adult exposed mice. **Reproductive Toxicology (Elmsford, N.Y.)**, v. 77, p. 70–79, abr. 2018.

SEACHRIST, D. D. et al. A review of the carcinogenic potential of bisphenol A. **Reproductive Toxicology (Elmsford, N.Y.)**, v. 59, p. 167–182, jan. 2016.

SHAFEI, A. et al. Stop eating plastic, molecular signaling of bisphenol A in breast cancer. **Environmental Science and Pollution Research International**, v. 25, n. 24, p. 23624–23630, 2018.

VAN LEEUWEN, F. E. et al. Risk of endometrial cancer after tamoxifen treatment of breast cancer. **Lancet**, v. 19, p. 448-452, 1994.

VARAYOUD, J. et al. Effects of a glyphosate-based herbicide on the uterus of adult ovariectomized rats. **Environ Toxicol**, v. 32, p. 1191-1201, Apr. 2017.

WALKER, C. L. Minireview: Epigenomic Plasticity and Vulnerability to EDC Exposures. **Molecular Endocrinology (Baltimore, Md.)**, v. 30, n. 8, p. 848–855, 2016.

ZAID, S. S. M. et al. Protective role of Mas Cotek (Ficus deltoidea) against the toxic effects of bisphenol A on morphology and sex steroid receptor expression in the rat uterus. **Biomed Phamacother**, Aug. 2021.

ZHOU C. et al. Prenatal exposure to an environmentally relevant phthalate mixture disrupts reproduction in F1 female mice.**Toxicol Appl Pharmacol**, v. 318, p. 49-57, 2017

.

ANEXO A - NORMAS DE SUBMISSÃO ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH

Submission guidelines

Environmental Science and Pollution Research

General Information

Note on preprint server:

Please add a note to the manuscript cover letter declaring whether you have submitted your manuscript to a preprint server. Please add one of the following sentences:

"I have submitted my manuscript to a preprint server before submitting it to *Environmental Science and Pollution Research*" or

"I have not submitted my manuscript to a preprint server before submitting it to *Environmental Science and Pollution Research*".

Types of Papers

Peer-reviewed contributions:

- Research Articles (full papers)
- Short Original Communications and Discussion Articles
- Review Articles
- Research Communications

Please ensure that the length of your paper is in harmony with your research area and with the science presented.

All papers – excluding Editorials, Letters to the Editor, Conference Reports – are subject to peer-review by a minimum of two and a maximum of three experts.

While submitting your paper you will be asked for three potential reviewers. Indicating three reviewers is mandatory.

- To authors from non-English language countries:

To have the best possible pre-requisition for the review process, please ask a native speaker to check the quality of the English, before you submit the complete paper.

Letters to the Editor

Please provide the following details within your manuscript:

- full title of the article you are commenting on
- corresponding author of the article you are commenting on
- DOI of the article that you are commenting on

The title of your letter should be structured as follows:

- Comments on "Title of the Article" by Corresponding Author's Last name, First name et al., DOI (e.g.: https://doi.org/10.1007/s11356...)

or

- Answer to "Comments on "Title of the Article" by Corresponding Author's Last name, First name et al., DOI (e.g.: https://doi.org/10.1007/s11356...)"

Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink "Submit manuscript" and upload all of your manuscript files following the

instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
  - Please avoid acronyms in the title of your article
  - For local studies, please indicate the name of the region and country in the title.
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of about 10 to 15 lines.

Keywords

Please provide 6 to 8 keywords which can be used for indexing purposes.

Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Additional Information Text Formatting

All manuscripts should be formatted containing continuous line numbering. Use the page and line numbering function to number the pages.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

Reference list entries should be alphabetized by the last names of the first author of each work. Please alphabetize according to the following rules: 1) For one author, by name of author, then chronologically; 2) For two authors, by name of author, then name of coauthor, then chronologically; 3) For more than two authors, by name of first author, then chronologically.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

  Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

  ISSN LTWA

  If you are unsure, please use the full journal title.

Specific Remarks

- Online documents:wikipedia documents are not acceptable as references.
- LanguageReferences should be in English with an appropriate title in English. If it’s in a different language the language should be indicatedZhu J, Wu F-C, Deng Q-J, Shao S-X, Mo C-L, Pan X-L, Li W, Zhang R-Y (2009) Environmental characteristics of water near the Xikuangshan antimony mine. Acta Scientiae Circumstantiae 29:655-661 (in Chinese)

Statements & Declarations

The following statements must be included in your submitted manuscript under the heading 'Statements and Declarations'. This should be placed after the References section. Please note that submissions that do not include required statements will be returned as incomplete.

Funding

Please describe any sources of funding that have supported the work. The statement should include details of any grants received (please give the name of the funding agency and grant number).

Example statements:

“This work was supported by […] (Grant numbers […] and […]). Author A.B. has received research support from Company A.”

“The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.”

Competing Interests

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work.

Example statements:

“Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and

consultant honoraria from Company M. Dr. C has received speaker honorarium and research funding from Company M and Company N. Author D has received travel support from Company O. Non-financial interests: Author D has served on advisory boards for Company M and Company N."
"The authors have no relevant financial or non-financial interests to disclose."
Please refer to the "Competing Interests" section below for more information on how to complete these sections.

Author Contributions

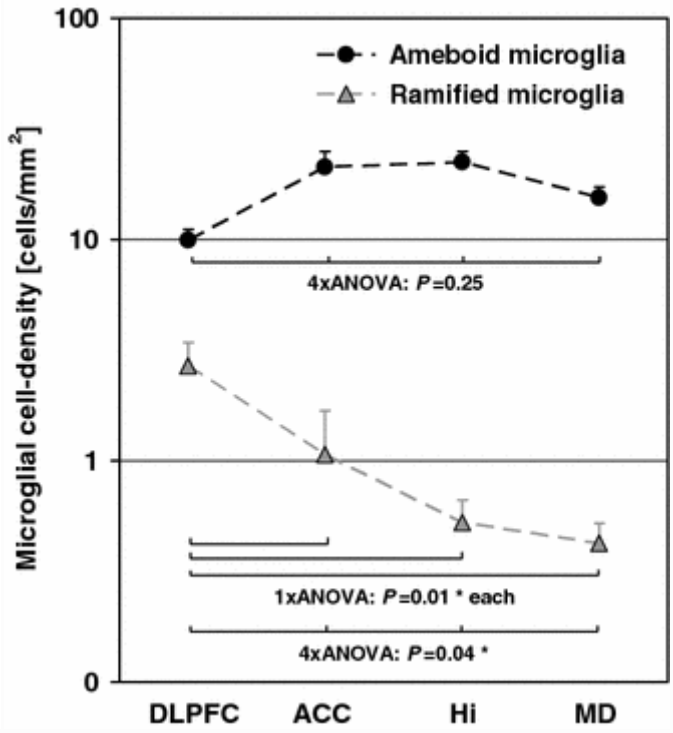
Authors are encouraged to include a statement that specifies the contribution of every author to the research and preparation of the manuscript.
Example statement:
"All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."
Please refer to the "Authorship Principles " section below for more information on how to complete this section.
Tables
- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork
For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

Electronic Figure Submission

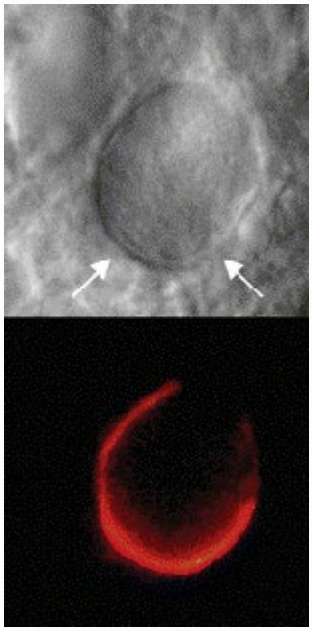
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



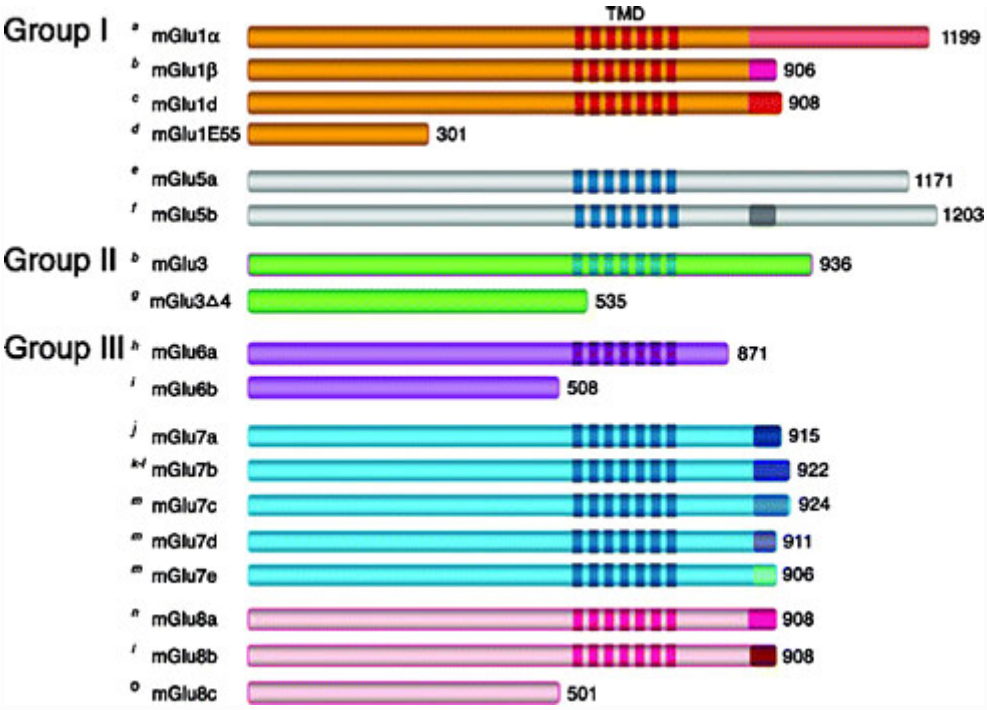
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for print and online publication.
- Color illustrations should be submitted as RGB.

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)
- Patterns are used instead of or in addition to colors for conveying information (color-blind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

Supplementary Information (SI)

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal's Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.
- High resolution (streamable quality) videos can be submitted up to a maximum of 25GB; low resolution videos should not be larger than 5GB.

Audio, Video, and Animations

- Aspect ratio: 16:9 or 4:3
- Maximum file size: 25 GB for high resolution files; 5 GB for low resolution files
- Minimum video duration: 1 sec
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

Text and Presentations

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as "Online Resource", e.g., "... as shown in the animation (Online Resource 3)", "... additional data are given in Online Resource 4".
- Name the files consecutively, e.g. "ESM_3.mpg", "ESM_4.pdf".

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

Editing Services

English

How can you help improve your manuscript for publication?

Presenting your work in a well-structured manuscript and in well-written English gives it its best chance for editors and reviewers to understand it and evaluate it fairly. Many researchers find that getting some independent support helps them present their results in the best possible light. The experts at Springer Nature Author Services can help you with manuscript preparation—including **English language editing, developmental comments, manuscript formatting, figure preparation, translation**, and more.

Get started and save 15%

You can also use our free Grammar Check tool for an evaluation of your work.

Please note that using these tools, or any other service, is not a requirement for publication, nor does it imply or guarantee that editors will accept the article, or even select it for peer review.

Chinese (中文)

您怎么做才有助于改进您的稿件以便顺利发表？

如果在结构精巧的稿件中用精心组织的英语展示您的作品，就能最大限度地让编辑和审稿人理解并公正评估您的作品。许多研究人员发现，获得一些独立支持有助于他们以尽可能美好的方式展示他们的成果。Springer Nature Author Services **的**专家可帮助您准备**稿件，具体包括润色英语表述、添加有见地的注释、为稿件排版、设计图表、翻译等**。

开始使用即可节省 15% **的**费用

您还可以使用我们的**免**费语法检查工具**来**评估您的作品。

请注意，使用这些工具或任何其他服务不是发表前必须满足的要求，也不暗示或保证相关文章定会被编辑接受（甚至未必会被选送同行评审）。

Japanese (日本語)

発表に備えて、論文を改善するにはどうすればよいでしょうか？

内容が適切に組み立てられ、質の高い英語で書かれた論文を投稿すれば、編集者や査読者が論文を理解し、公正に評価するための最善の機会となります。多くの研究者は、個別のサポートを受けることで、研究結果を可能な限り最高の形で発表できると思っています。Springer Nature Author Services**のエキスパートが、英文の編集、建設的な提言、論文の書式、図の調整、翻訳**など、論文の作成をサポートいたします。

今なら15%割引でご利用いただけます

原稿の評価に、無料の文法チェック**ツールもご利用いただけます。**

これらのツールや他のサービスをご利用いただくことは、論文を掲載するための要件ではありません。また、編集者が論文を受理したり、査読に選定したりすることを示唆または保証するものではないことにご注意ください。

Korean (한국어)

게재를 위해 원고를 개선하려면 어떻게 해야 할까요?

여러분의 작품을 체계적인 원고로 발표하는 것은 편집자와 심사자가 여러분의 연구를 이해하고 공정하게 평가할 수 있는 최선의 기회를 제공합니다. 많은 연구자들은 어느 정도 독립적인 지원을 받는 것이 가능한 한 최선의 방법으로 자신의 결과를 발표하는 데 도움이 된다고 합니다. Springer Nature Author Services 전문가들은 **영어 편집, 발전적인 논평, 원고 서식 지정, 그림 준비, 번역** 등과 같은 원고 준비를 도와드릴 수 있습니다.

지금 시작하면 15% 할인됩니다.

또한 당사의 무료 문법 검사도구를 사용하여 여러분의 연구를 평가할 수 있습니다.

이러한 도구 또는 기타 서비스를 사용하는 것은 게재를 위한 필수 요구사항이 아니며, 편집자가 해당 논문을 수락하거나 피어 리뷰에 해당 논문을 선택한다는 것을 암시하거나 보장하지는 않습니다.

Ethical Responsibilities of Authors

This journal is committed to upholding the integrity of the scientific record. As a member of the Committee on Publication Ethics (COPE) the journal will follow the COPE guidelines on how to deal with potential acts of misconduct.

Authors should refrain from misrepresenting research results which could damage the trust in the journal, the professionalism of scientific authorship, and ultimately the entire scientific endeavour. Maintaining integrity of the research and its presentation is helped by following the rules of good scientific practice, which include*:

- The manuscript should not be submitted to more than one journal for simultaneous consideration.
- The submitted work should be original and should not have been published elsewhere in any form or language (partially or in full), unless the new work concerns an expansion of previous work. (Please provide transparency on the re-use of material to avoid the concerns about text-recycling ('self-plagiarism').
- A single study should not be split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (i.e. 'salami-slicing/publishing').
- Concurrent or secondary publication is sometimes justifiable, provided certain conditions are met. Examples include: translations or a manuscript that is intended for a different group of readers.
- Results should be presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation). Authors should adhere to discipline-specific rules for acquiring, selecting and processing data.
- No data, text, or theories by others are presented as if they were the author's own ('plagiarism'). Proper acknowledgements to other works must be given (this includes material that is closely copied (near verbatim), summarized and/or paraphrased), quotation marks (to indicate words taken from another source) are used for verbatim copying of material, and permissions secured for material that is copyrighted.

Important note: the journal may use software to screen for plagiarism.

- Authors should make sure they have permissions for the use of software, questionnaires/(web) surveys and scales in their studies (if appropriate).
- Research articles and non-research articles (e.g. Opinion, Review, and Commentary articles) must cite appropriate and relevant literature in support of the claims made. Excessive and inappropriate self-citation or coordinated efforts among several authors to collectively self-cite is strongly discouraged.
- Authors should avoid untrue statements about an entity (who can be an individual person or a company) or descriptions of their behavior or actions that could potentially be seen as personal attacks or allegations about that person.
- Research that may be misapplied to pose a threat to public health or national security should be clearly identified in the manuscript (e.g. dual use of research). Examples include creation of harmful consequences of biological agents or toxins, disruption of immunity of vaccines, unusual hazards in the use of chemicals, weaponization of research/technology (amongst others).
- Authors are strongly advised to ensure the author group, the Corresponding Author, and the order of authors are all correct at submission. Adding and/or deleting authors during the revision stages is generally not permitted, but in some cases may be warranted. Reasons for changes in authorship should be explained in detail. Please note that changes to authorship cannot be made after acceptance of a manuscript.

*All of the above are guidelines and authors need to make sure to respect third parties rights such as copyright and/or moral rights.

Upon request authors should be prepared to send relevant documentation or data in order to verify the validity of the results presented. This could be in the form of raw data, samples, records, etc. Sensitive information in the form of confidential or proprietary data is excluded.

If there is suspicion of misbehavior or alleged fraud the Journal and/or Publisher will carry out an investigation following COPE guidelines. If, after investigation, there are valid concerns, the author(s) concerned will be contacted under their given e-mail address and given an opportunity to address the issue. Depending on the situation, this may result in the Journal's and/or Publisher's implementation of the following measures, including, but not limited to:

- If the manuscript is still under consideration, it may be rejected and returned to the author.
- If the article has already been published online, depending on the nature and severity of the infraction:

- an erratum/correction may be placed with the article
- an expression of concern may be placed with the article
- or in severe cases retraction of the article may occur.

The reason will be given in the published erratum/correction, expression of concern or retraction note. Please note that retraction means that the article is **maintained on the platform**, watermarked "retracted" and the

explanation for the retraction is provided in a note linked to the watermarked article.

- The author's institution may be informed
- A notice of suspected transgression of ethical standards in the peer review system may be included as part of the author's and article's bibliographic record.

Fundamental errors

Authors have an obligation to correct mistakes once they discover a significant error or inaccuracy in their published article. The author(s) is/are requested to contact the journal and explain in what sense the error is impacting the article. A decision on how to correct the literature will depend on the nature of the error. This may be a correction or retraction. The retraction note should provide transparency which parts of the article are impacted by the error.

Suggesting / excluding reviewers

Authors are welcome to suggest suitable reviewers and/or request the exclusion of certain individuals when they submit their manuscripts. When suggesting reviewers, authors should make sure they are totally independent and not connected to the work in any way. It is strongly recommended to suggest a mix of reviewers from different countries and different institutions. When suggesting reviewers, the Corresponding Author must provide an institutional email address for each suggested reviewer, or, if this is not possible to include other means of verifying the identity such as a link to a personal homepage, a link to the publication record or a researcher or author ID in the submission letter. Please note that the Journal may not use the suggestions, but suggestions are appreciated and may help facilitate the peer review process.

Please note: The suggested reviewers must not be from the same university nor laboratory and should not have shared authorship in previous publications.

Authorship principles

These guidelines describe authorship principles and good authorship practices to which prospective authors should adhere to.

Authorship clarified

The Journal and Publisher assume all authors agreed with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute/organization where the work has been carried out, **before** the work is submitted.

The Publisher does not prescribe the kinds of contributions that warrant authorship. It is recommended that authors adhere to the guidelines for authorship that are applicable in their specific research field. In absence of specific guidelines it is recommended to adhere to the following guidelines*:

All authors whose names appear on the submission

1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;

2) drafted the work or revised it critically for important intellectual content;

3) approved the version to be published; and

4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,

Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt at all, PNAS February 27, 2018

Disclosures and declarations

All authors are requested to include information regarding sources of funding, financial or non-financial interests, study-specific approval by the appropriate ethics committee for research involving humans and/or animals, informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals (as appropriate).

The decision whether such information should be included is not only dependent on the scope of the journal, but also the scope of the article. Work submitted for publication may have implications for public health or general welfare and in those cases it is the responsibility of all authors to include the appropriate disclosures and declarations.

Data transparency

All authors are requested to make sure that all data and materials as well as software application or custom code support their published claims and comply with field standards. Please note that journals may have individual policies on (sharing) research data in concordance with disciplinary norms and expectations.

Role of the Corresponding Author

One author is assigned as Corresponding Author and acts on behalf of all co-authors and ensures that

questions related to the accuracy or integrity of any part of the work are appropriately addressed.
The Corresponding Author is responsible for the following requirements:
• ensuring that all listed authors have approved the manuscript before submission, including the names and order of authors;
• managing all communication between the Journal and all co-authors, before and after publication;*
• providing transparency on re-use of material and mention any unpublished material (for example manuscripts in press) included in the manuscript in a cover letter to the Editor;
• making sure disclosures, declarations and transparency on data statements from all authors are included in the manuscript as appropriate (see above).
* The requirement of managing all communication between the journal and all co-authors during submission and proofing may be delegated to a Contact or Submitting Author. In this case please make sure the Corresponding Author is clearly indicated in the manuscript.

Author contributions

In absence of specific instructions and in research fields where it is possible to describe discrete efforts, the Publisher recommends authors to include contribution statements in the work that specifies the contribution of every author in order to promote transparency. These contributions should be listed at the separate title page.

Examples of such statement(s) are shown below:

• Free text:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Example: CRediT taxonomy:

• Conceptualization: [full name], …; Methodology: [full name], …; Formal analysis and investigation: [full name], …; Writing - original draft preparation: [full name, …]; Writing - review and editing: [full name], …; Funding acquisition: [full name], …; Resources: [full name], …; Supervision: [full name],….

For **review articles** where discrete statements are less applicable a statement should be included who had the idea for the article, who performed the literature search and data analysis, and who drafted and/or critically revised the work.

For articles that are based primarily on the **student's dissertation or thesis**, it is recommended that the student is usually listed as principal author:

A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

Affiliation

The primary affiliation for each author should be the institution where the majority of their work was done. If an author has subsequently moved, the current address may additionally be stated. Addresses will not be updated or changed after publication of the article.

Changes to authorship

Authors are strongly advised to ensure the correct author group, the Corresponding Author, and the order of authors at submission. Changes of authorship by adding or deleting authors, and/or changes in Corresponding Author, and/or changes in the sequence of authors are **not** accepted **after acceptance** of a manuscript.

- **Please note that author names will be published exactly as they appear on the accepted submission!**

Please make sure that the names of all authors are present and correctly spelled, and that addresses and affiliations are current.

Adding and/or deleting authors at revision stage are generally not permitted, but in some cases it may be warranted. Reasons for these changes in authorship should be explained. Approval of the change during revision is at the discretion of the Editor-in-Chief. Please note that journals may have individual policies on adding and/or deleting authors during revision stage.

Author identification

Authors are recommended to use their ORCID ID when submitting an article for consideration or acquire an ORCID ID via the submission process.

Deceased or incapacitated authors

For cases in which a co-author dies or is incapacitated during the writing, submission, or peer-review process, and the co-authors feel it is appropriate to include the author, co-authors should obtain approval from a (legal) representative which could be a direct relative.

Authorship issues or disputes

In the case of an authorship dispute during peer review or after acceptance and publication, the Journal will not be in a position to investigate or adjudicate. Authors will be asked to resolve the dispute themselves. If they are unable the Journal reserves the right to withdraw a manuscript from the editorial process or in case of a published paper raise the issue with the authors' institution(s) and abide by its guidelines.

Confidentiality

Authors should treat all communication with the Journal as confidential which includes correspondence with direct representatives from the Journal such as Editors-in-Chief and/or Handling Editors and reviewers' reports unless explicit consent has been received to share information.

Important note:

Please note that – contrary to the text above – **according to the policy of ESPR, any addition/removal of authors or change in order list are prohibited during all the review process. The original authorship must remain the same during all the review process.**

Compliance with Ethical Standards

To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, authors should include information regarding sources of funding, potential conflicts of interest (financial or non-financial), informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals.

Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
- Research involving Human Participants and/or Animals
- Informed consent

Please note that standards could vary slightly per journal dependent on their peer review policies (i.e. single or double blind peer review) as well as per journal subject discipline. Before submitting your article check the instructions following this section carefully.

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

The Editors reserve the right to reject manuscripts that do not comply with the above-mentioned guidelines. The author will be held responsible for false statements or failure to fulfill the above-mentioned guidelines.

Competing Interests

Authors are requested to disclose interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work. Disclosure of interests provides a complete and transparent process and helps readers form their own judgments of potential bias. This is not meant to imply that a financial relationship with an organization that sponsored the research or compensation received for consultancy work is inappropriate.

Editorial Board Members and Editors are required to declare any competing interests and may be excluded from the peer review process if a competing interest exists. In addition, they should exclude themselves from handling manuscripts in cases where there is a competing interest. This may include – but is not limited to – having previously published with one or more of the authors, and sharing the same institution as one or more of the authors. Where an Editor or Editorial Board Member is on the author list they must declare this in the competing interests section on the submitted manuscript. If they are an author or have any other competing interest regarding a specific manuscript, another Editor or member of the Editorial Board will be assigned to assume responsibility for overseeing peer review. These submissions are subject to the exact same review process as any other manuscript. Editorial Board Members are welcome to submit papers to the journal. These submissions are not given any priority over other manuscripts, and Editorial Board Member status has no bearing on editorial consideration.

Interests that should be considered and disclosed but are not limited to the following:

Funding: Research grants from funding agencies (please give the research funder and the grant number) and/or research support (including salaries, equipment, supplies, reimbursement for attending symposia, and other expenses) by organizations that may gain or lose financially through publication of this manuscript.

Employment: Recent (while engaged in the research project), present or anticipated employment by any organization that may gain or lose financially through publication of this manuscript. This includes multiple affiliations (if applicable).

Financial interests: Stocks or shares in companies (including holdings of spouse and/or children) that may gain or lose financially through publication of this manuscript; consultation fees or other forms of remuneration

from organizations that may gain or lose financially; patents or patent applications whose value may be affected by publication of this manuscript.
It is difficult to specify a threshold at which a financial interest becomes significant, any such figure is necessarily arbitrary, so one possible practical guideline is the following: "Any undeclared financial interest that could embarrass the author were it to become publicly known after the work was published."
Non-financial interests: In addition, authors are requested to disclose interests that go beyond financial interests that could impart bias on the work submitted for publication such as professional interests, personal relationships or personal beliefs (amongst others). Examples include, but are not limited to: position on editorial board, advisory board or board of directors or other type of management relationships; writing and/or consulting for educational purposes; expert witness; mentoring relations; and so forth.
Primary research articles require a disclosure statement. Review articles present an expert synthesis of evidence and may be treated as an authoritative work on a subject. Review articles therefore require a disclosure statement.Other article types such as editorials, book reviews, comments (amongst others) may, dependent on their content, require a disclosure statement. If you are unclear whether your article type requires a disclosure statement, please contact the Editor-in-Chief.
Please note that, in addition to the above requirements, funding information (given that funding is a potential competing interest (as mentioned above)) needs to be disclosed upon submission of the manuscript in the peer review system. This information will automatically be added to the Record of CrossMark, however it is **not added** to the manuscript itself. Under 'summary of requirements' (see below) funding information should be included in the '**Declarations**' section.
Summary of requirements
The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Funding' and/or 'Competing interests'. Other declarations include Ethics approval, Consent, Data, Material and/or Code availability and Authors' contribution statements.
Please see the various examples of wording below and revise/customize the sample statements according to your own needs.
When all authors have the same (or no) conflicts and/or funding it is sufficient to use one blanket statement.
Examples of statements to be used when funding has been received:

- Partial financial support was received from [...]
- The research leading to these results received funding from […] under Grant Agreement No[…].
- This study was funded by […]
- This work was supported by […] (Grant numbers […] and […]

Examples of statements to be used when there is no funding:

- The authors did not receive support from any organization for the submitted work.
- No funding was received to assist with the preparation of this manuscript.
- No funding was received for conducting this study.
- No funds, grants, or other support was received.

Examples of statements to be used when there are interests to declare:

- **Financial interests:** Author A has received research support from Company A. Author B has received a speaker honorarium from Company Wand owns stock in Company X. Author C is consultant to company Y.

Non-financial interests: Author C is an unpaid member of committee Z.

- **Financial interests:** The authors declare they have no financial interests.

Non-financial interests: Author A is on the board of directors of Y and receives no compensation as member of the board of directors.

- **Financial interests:** Author A received a speaking fee from Y for Z. Author B receives a salary from association X. X where s/he is the Executive Director.

Non-financial interests: none.

- **Financial interests:** Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M and Company N. Dr. C has received speaker honorarium and research funding from Company M and Company O. Author D has received travel support from Company O.

Non-financial interests: Author D has served on advisory boards for Company M, Company N and Company O.
Examples of statements to be used when authors have nothing to declare:

- The authors have no relevant financial or non-financial interests to disclose.
- The authors have no competing interests to declare that are relevant to the content of this article.
- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

- The authors have no financial or proprietary interests in any material discussed in this article.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research Data Policy

This journal operates a type 1 research data policy. The journal encourages authors, where possible and applicable, to deposit data that support the findings of their research in a public repository. Authors and editors who do not have a preferred repository should consult Springer Nature's list of repositories and research data policy.

List of Repositories

Research Data Policy

General repositories - for all types of research data - such as figshare and Dryad may also be used.

Datasets that are assigned digital object identifiers (DOIs) by a data repository may be cited in the reference list. Data citations should include the minimum information recommended by DataCite: authors, title, publisher (repository name), identifier.

DataCite

If the journal that you're submitting to uses double-blind peer review and you are providing reviewers with access to your data (for example via a repository link, supplementary information or data on request), it is strongly suggested that the authorship in the data is also blinded. There are data repositories that can assist with this and/or will create a link to mask the authorship of your data.

Authors who need help understanding our data sharing policies, help finding a suitable data repository, or help organising and sharing research data can access our Author Support portal for additional guidance.

After Acceptance

Upon acceptance, your article will be exported to Production to undergo typesetting. Once typesetting is complete, you will receive a link asking you to confirm your affiliation, choose the publishing model for your article as well as arrange rights and payment of any associated publication cost.

Once you have completed this, your article will be processed and you will receive the proofs.

Article publishing agreement

Depending on the ownership of the journal and its policies, you will either grant the Publisher an exclusive licence to publish the article or will be asked to transfer copyright of the article to the Publisher.

Offprints

Offprints can be ordered by the corresponding author.

Color illustrations

Publication of color illustrations is free of charge.

Proof reading

The purpose of the proof is to check for typesetting or conversion errors and the completeness and accuracy of the text, tables and figures. Substantial changes in content, e.g., new results, corrected values, title and authorship, are not allowed without the approval of the Editor.

After online publication, further changes can only be made in the form of an Erratum, which will be hyperlinked to the article.

Online First

The article will be published online after receipt of the corrected proofs. This is the official first publication citable with the DOI. After release of the printed version, the paper can also be cited by issue and page numbers.

Open Choice

Open Choice allows you to publish open access in more than 1850 Springer Nature journals, making your research more visible and accessible immediately on publication.

Article processing charges (APCs) vary by journal – view the full list

Benefits:

- Increased researcher engagement: Open Choice enables access by anyone with an internet connection, immediately on publication.
- Higher visibility and impact: In Springer hybrid journals, OA articles are accessed 4 times more often on average, and cited 1.7 more times on average*.
- Easy compliance with funder and institutional mandates: Many funders require open access publishing, and some take compliance into account when assessing future grant applications.

It is easy to find funding to support open access – please see our funding and support pages for more information.

*) Within the first three years of publication. Springer Nature hybrid journal OA impact analysis, 2018.
Open Choice
Funding and Support pages

ANEXO B - PARECER FINAL

21/03/2022 18:53 Certificado

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPD - Projeto de Pesquisa Docente

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado "CARACTERIZAÇÃO DA TRÍADE EXPOSIÇÃO-GENE-DOENÇA: EFEITOS DE UMA MISTURA DE DESREGULADORES ENDÓCRINOS BASEADA NA EXPOSIÇÃO HUMANA SOBRE O MICROAMBIENTE PROSTÁTICO.", cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº 6834 e tendo como participante(s) MARIA LUIZA SILVA RICARDO (discente), WELLERSON RODRIGO SCABARO (participante externo), ANTHONY CESAR DE SOUZA CASTILHO (discente), LEONARDO DE OLIVEIRA SEPEDRO (orientador responsável), foi avaliado e APROVADO pelo COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA) da Universidade do Oeste Paulista – UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Cordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com as 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 de Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 11/05/2020.

Vigência do projeto: 02/2020 a 11/2023.

ANIMAL VIVO

Espécie/Linhagem	Nº de Animais	Peso	Idade	Sexo	Origem
Ratos Sprague-Dawley	36	250 gramas	60 dias	F	CEMIB - UNICAMP
Ratos Sprague-Dawley	12	300 gramas	90 dias	M	CEMIB - UNICAMP

Presidente Prudente, 2 de Abril de 2020.

Prof. Dr. João Rodrigues Garcia Jr.
Coordenador Científico do CPDI

Profª Ms. Adriana Pelaio de Brito
Coordenadora do CEUA - UNOESTE

Coordenadoria de Pesquisa, Desenvolvimento e Inovação – CPDI – 18 3229-2072 – cpdi@unoeste.br
Comitê de Ética em Pesquisa – CEP – 18 3229-2070 – cep@unoeste.br
Comissão de Ética no Uso de Animais – CEUA – 18 3229-2073 – ceua@unoeste.br

Valide este documento em www.unoeste.br/cgi-bin/validadoc informando o código de segurança xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx