SUELLEN RIBEIRO DA SILVA SCARTON

A EXPOSIÇÃO AO CYANTRANILIPROLE PREJUDICA PARÂMETROS REPRODUTIVOS MASCULINOS NA IDADE JUVENIL E PARÂMETROS FEMININOS NA IDADE ADULTA EM RATOS WISTAR

Londrina
2021

SUELLEN RIBEIRO DA SILVA SCARTON

A EXPOSIÇÃO AO CYANTRANILIPROLE PREJUDICA PARÂMETROS REPRODUTIVOS MASCULINOS NA IDADE JUVENIL E PARÂMETROS FEMININOS NA IDADE ADULTA EM RATOS WISTAR

Tese apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutora.

Orientadora: Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coorientadora: Profa. Dra. Célia Cristina Leme Beu

Londrina
2021

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

S287 Scarton, Suellen Ribeiro da.
A EXPOSIÇÃO AO CYANTRANILIPROLE PREJUDICA PARÂMETROS REPRODUTIVOS MASCULINOS NA IDADE JUVENIL E PARÂMETROS FEMININOS NA IDADE ADULTA EM RATOS WISTAR / Suellen Ribeiro da Scarton. - Londrina, 2021.
140 f. : il.

Orientador: Glaura Scantamburlo Alves Fernandes.
Coorientador: Célia Cristina Leme Beu.
Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2021.
Inclui bibliografia.

1. Inseticida - Tese. 2. Estresse oxidativo - Tese. 3. Puberdade - Tese. 4. Reprodução - Tese. I. Fernandes, Glaura Scantamburlo Alves. II. Beu, Célia Cristina Leme. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. IV. Título.

CDU 616

SUELLEN RIBEIRO DA SILVA SCARTON

A EXPOSIÇÃO AO CYANTRANILIPROLE PREJUDICA PARÂMETROS REPRODUTIVOS MASCULINOS NA IDADE JUVENIL E PARÂMETROS FEMININOS NA IDADE ADULTA EM RATOS WISTAR

Tese apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutora.

BANCA EXAMINADORA

_____

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

_____

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

_____

Profa. Dra. Arielle Cristina Arena
Universidade Estadual Paulista – UNESP

_____

Prof. Dra. Graziela Scalianti Ceravolo
Universidade Estadual de Londrina - UEL

_____

Profa. Dra. Rejane Maira Goes
Universidade Estadual Paulista – UNESP

Londrina, 25 de junho de 2021.

Ao meu marido, amigo e companheiro, com quem divido as alegrias e as tristezas... agradeço por decidir estar presente, juntos vencemos!

“A distância faz ao amor aquilo que o vento faz ao fogo; apaga o pequeno e inflama o grande”.

Roger de Bussy-Rabutin

AGRADECIMENTOS

Os maiores e mais sinceros agradecimentos sempre serão para a minha família, lugar onde meus sonhos semeados florescem, onde o apoio e carinho se fazem presentes. Obrigada minha mãe, Dona Sueli, pelas orações e por suas palavras que reforçavam a minha fé a cada vez que de casa eu saia, em muitos momentos, essa foi a força que me manteve confiante. Não há como não deixar aqui registrado todo o esforço feito por você, amor... Leandro, quantos quilômetros percorridos, quantas horas ao telefone foram gastas durante os dois anos em que eu estive fora de casa? Foram muitos e muitos... Só posso te agradecer por entender e aceitar junto comigo os desafios e principalmente as incertezas da vida acadêmica. Quem iria garantir que eu teria como me manter enquanto estudava? Ou que eu teria um emprego após terminar o doutorado? Não tínhamos garantias... e apesar de tudo, nós continuamos, não era só um sonho, é o que me faz feliz, e eu sei que você abriu mão de muitas coisas para que eu pudesse estar aqui hoje, nem todas as palavras do mundo seriam suficientes para explicar o quanto obrigada! Nas muitas idas e vindas de Cascavel à Londrina, outra pessoa esteve quase sempre no banco do carona, tia Deja, mais uma vez nos apoiando e fortalecendo a nossa família. Agradeço por todas as palavras encorajadoras e por se fazer tão presente em nossas vidas, mais do que isso, agradeço pelo exemplo de vida e superação, acredito que são esses exemplos que me fizeram ter forças para ir sempre além.

É preciso agradecer aquelas pessoas que muitas vezes acreditaram muito mais em mim do que eu mesma, Professora Dra. Célia Cristina Leme Beu, minha coorientadora, e Professora Dra. Lucinéia Chasko Ribeiro, que me conduziram a esse mundo maravilhoso da ciência e nunca me deixaram sozinha, uma amizade que saiu dos laboratórios e foi para vida. Obrigada por todo cuidado, carinho e compreensão que sempre dedicaram a mim, espero um dia poder retribuir a altura. Amo vocês!

Agradeço a minha orientadora Professora Dra. Glaura Scantamburlo Alves Fernandes, por ter me aceitado, mesmo não me conhecendo, e por ter dedicado o tempo necessário para que eu me tornasse não só uma profissional melhor, mas por ajudar a me transformar numa pessoa melhor. Em seu nome agradeço a todos os professores do Programa de Pós Graduação em Patologia Experimental, assim como a senhora, cada um deixou um pouquinho de si em mim. Foram momentos memoráveis que serão guardados com muito carinho em meu coração! Eu espero que cada um de vocês tenha a real noção de quão importantes são para seus alunos, vocês transformam vidas!

Para que esse trabalho fosse realizado, Professores que não fazem parte no nosso PGG também se empenharam muito, Professora Dra. Sabrina Grassiolli – Universidade Estadual do Oeste Paraná, pela qual recebemos todos os animais machos utilizados em nosso experimento; Professora Dra. Marina Trevizan Guerra – Universidade Federal do Mato

Grosso do Sul, nos conduziu na realização do experimento com fêmeas; Professora Dra. Ana Tereza Bittencourt Guimarães – Universidade Estadual do Oeste do Paraná, nos auxiliou com todos os experimentos de estresse oxidativo. Obrigada por acreditarem e apoiarem a realização deste trabalho. Agradeço as professoras que compõe a banca avaliadora desse trabalho, Prof. Dra. Alessandra Lourenço Cecchini Armani - Universidade Estadual de Londrina, Profa. Dra. Arielle Cristina Arena - Universidade Estadual Paulista "Júlio de Mesquita Filho", Prof. Dra. Graziela Scalianti Ceravolo - Universidade Estadual de Londrina, Profa. Dra. Rejane Maira Goes - Universidade Estadual Paulista "Júlio de Mesquita Filho", que disponibilizaram o seu tempo e conhecimento para torná-lo melhor, a escolha de cada uma das Senhoras foi pensada com muito carinho e é uma honra tê-las comigo nesse momento tão especial.

Por fim, em nenhum lugar do mundo se faz ciência sozinho! Seja pelo apoio emocional ou pela força de trabalho muitas pessoas são necessárias, e que sorte a minha... durante todos esses anos estive rodeada de pessoas que além de extremamente capacitadas e terem um imenso coração, já sinto a falta de cada um de vocês! Lívia minha companheira, juntas vivemos as alegrias da vida e as dores que a morte trás... obrigada por sua amizade e paciência! Felipe, Dayane e Rafaela esse trabalho é tão meu quanto de vocês, foram longos 8 meses de experimento em biotério e eu não teria conseguido sem a ajuda e a disponibilidade de cada um. Obrigada por todos os fins de semana e pelos dias de coleta que dedicaram a esse trabalho. Vocês são incríveis! Sempre estarão em meu coração! Para quem tem duas casas, os agradecimentos são longos. Como foi bom voltar e encontrar todos novamente, Ana Caroline, Aldair, Matheus e Fernando. Se na UEL eu encontrei novas amizades, ao voltar para a UNIOESTE realmente entendi que a distância e o tempo que nos manteve afastados não diminuiu a importância de cada um de vocês na minha vida! Obrigada por toda a ajuda na realização das análises, pela companhia e pelos momentos de lazer.

Sinto um orgulho imenso em dizer que sou aluna da Universidade Estadual de Londrina e do Programa de Pós Graduação em Patologia Experimental, estar aqui foi um sonho que agora se completa plenamente e sinceramente estou sentindo um misto de alegrias e tristezas inexplicáveis, é difícil dizer adeus! Mas tudo que temos quando se inicia uma jornada é a certeza de que um dia ela acaba, então muito obrigada. Agradeço a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES

e a Pró-Reitoria de Extensão, Cultura e Sociedade – PROEX, pelo apoio financeiro de extrema valia nesse período, sem essa condição, talvez eu não estivesse aqui.

Por todos os momentos e oportunidades vividas durante esse período eu te agradeço meu Deus, não poderia ter sido melhor!

“Mais bonito que o canto dos pássaros, são seus voos.
Nem todo canto é de alegria, mas todo voo é de liberdade.”

Mario Quintana

SCARTON, Suellen Ribeiro da Silva. **A exposição ao *cyantraniliprole* prejudica parâmetros reprodutivos masculinos na idade juvenil e parâmetros femininos na idade adulta em ratos wistar**. 2021. 140 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

O Brasil é um dos países que mais utiliza agrotóxicos no mundo e conta com legislação favorável a liberação de compostos de ação pouco conhecida sobre o organismo de mamíferos ou banidos em outras regiões do mundo. O objetivo desse trabalho foi avaliar o efeito do inseticida Cyantraniliprole sobre parâmetros reprodutivos masculino e feminino de ratos Wistar púberes e adultos, após autorização OF. CIRC. CEUA nº134/2017, processo n° CEUA-UEL 211.062017-4. Em todos os experimentos o Cyantraniliprole foi utilizado em baixa dose de 10 mg / kg (grupo Low dose – LD) ou alta dose 150 mg / kg (grupo High dose – HD), água de torneira foi utilizada como veículo. No primeiro experimento machos, no dia pós-natal (DPN) 21, foram distribuídos nos grupos LD, HD ou controle (C), que recebeu somente veículo. O exposição se iniciou no DPN25, via intragástrica e a eutanásia ocorreu por saturação anestésica no DPN67. Espermatozoides foram coletados do ducto deferente para as análises de atividade mitocondrial da peça intermediaria, atividade da colinesterase – ChE, peroxidação lipídica – LPO, atividade da glicose-6-fosfato desidrogenase - G6PD e atividade da malato desidrogenase – MDH. No segundo experimento machos, DPN21, foram distribuídos nos grupos exposição: LD, HD e C. O tratamento se iniciou no DPN25, via intragástrica. A eutanásia de metade dos animais de cada grupo ocorreu por saturação anestésica no DPN67. Os outros animais de cada grupo foram mantidos vivos por mais 42 dias, sem receber o cyantraniliprole, originando os grupos recuperação, a eutanásia desses animais ocorreu por saturação anestésica no DPN110. De todos os grupos foram coletados plasma para determinação da concentração de testosterona. Os testículos foram pesados e utilizados para contagem espermática, avaliação do sistema antioxidante e de parâmetros histológicos e estereológicos. Espermatozoides do ducto deferente foram utilizados para avalição morfológica. No terceiro experimento, os efeitos do cyantraniliprole foram observados em fêmeas adultas, DPN67, distribuídas nos grupos C, LD e HD (n=6 por grupo). A eutanásia ocorreu no DPN95, foram coletados sangue, para dosagem de progesterona, útero e ovários para avaliação morfométrica e histopatológica, contagem de folículos ovarianos e marcadores bioquímicos de estresse oxidativo. Foi realizada a mensuração da massa (g) do fígado, rins, glândulas hipófise e adrenal. A concentração de progesterona e o peso do útero, ovário, fígado, rins, glândula adrenal e a foram maiores em LD. O número de ciclos estrais diminuiu e a duração do ciclo aumentou nos animais HD. O epitélio glandular do útero foi maior e o número de folículos ovarianos, primordial + primário, pré-antral, antral e atrésico foi reduzido em LD. A análise histopatológica revelou a ocorrência de metaplasia do epitélio luminal do útero com presença de mitose e polimorfismo das células do epitélio glandular, além da presença de infiltrado inflamatório no endométrio. Nos ovários, vasos sanguíneos congestos e hiperplasia da rete ovarii foram observados. A atividade da colinesterase no útero foi menor em ambas as doses. Maior ocorrência de lipoperoxidação e da atividade da enzima SOD ocorreram apenas em LD, e a atividade da GST foi aumentada em LD e HD. No ovário, a atividade da colinesterase

foi aumentada nos animais tratados com ambas as doses, e a atividade da GPx foi maior apenas com LD. Como resultados observamos que no primeiro experimento a atividade mitocondrial e da MDH diminuíram nos espermatozoides expostos a LD, sem o aumento da peroxidação lipídica. A atividade da G6PDH e da ChE foram similar entre os grupos. No segundo experimento a atividade do sistema antioxidante entre os grupos exposição e recuperação foi similar, no entanto a produção de testosterona in vitro aumentou em LD e HD e diminuiu em LDR e HDR. O número de células de Sertoli foi menor em todos os grupos que receberam cyantraniliprole tanto na fase de exposição como na recuperação. A fase I-V do ciclo do epitélio seminífero foi aumentada e a fase VII-VIII, diminuída nos grupos que receberam o tratamento nos dois períodos. A proporção do lúmen e estroma foi maior, e do epitélio menor, apenas em LD, no entanto o número de espermatozoides por grama de testículo e a produção diária de espermatozoides foi diminuiu nos animais que receberam ambas as doses de cyantraniliprole, independente da fase do experimento. Como consequência o número de espermatozoides normais foi menor nos grupos que receberam Cyantraniliprole em relação ao controle. As principais anormalidades morfológicas observadas nos espermatozoides foram: falta de curvatura e cabeças isoladas, caudas quebradas e enroladas, e falta de integridade do acrossoma. No terceiro experimento, a concentração de progesterona e o peso do útero, ovário, fígado, rins, glândula adrenal foram maiores em LD. O número de ciclos estrais diminuiu e a duração do ciclo aumentou nos animais HD. O epitélio glandular do útero foi maior e o número de folículos ovarianos, primordial + primário, pré-antral, antral e atrésico foi reduzido em LD. A análise histopatológica revelou a ocorrência de metaplasia do epitélio luminal do útero com presença de mitose e polimorfismo das células do epitélio glandular, além da presença de infiltrado inflamatório no endométrio. Nos ovários, vasos sanguíneos congestos e hiperplasia da rete ovarii foram observados. A atividade da colinesterase no útero foi menor em ambas as doses. Maior ocorrência de lipoperoxidação e da atividade da enzima SOD ocorreram apenas em LD, e a atividade da GST foi aumentada em LD e HD. No ovário, a atividade da colinesterase foi maior nos animais tratados com ambas as doses, e a atividade da GPx foi maior apenas com LD. Esses resultados mostram que a baixa dose de cyantraniliprole prejudicou a membrana celular e a produção de energia dos espermatozoides e que os danos gerados pela exposição ao cyantraniliprole na puberdade foram mantidos ou acentuados, mesmo após o período de recuperação, ou seja, na vida adulta. O trato genital feminino também sofreu alterações, o que pode representar prejuízos fertilidade a esses indivíduos.

Palavras-chave: inseticida; estresse oxidativo; puberdade; reprodução.

SCARTON, Suellen Ribeiro da Silva. **Exposure to cyantraniliprole hamps male reproductive parameters in the youth age and female parameters in the adult age in wistar rats.** 2021. 140 p. Thesis (Doctorate in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Brazil is one of the countries that most uses pesticides in the world and has legislation favorable to the release of compounds with little known action on the organism of mammals or banned in other regions of the world. The objective of this work was to evaluate the effect of the insecticide Cyantraniliprole on male and female reproductive parameters of pubescent and adult Wistar rats, after OP authorization. CIRCUIT CEUA No. 134/2017, case No. CEUA-UEL 211.062017-4. In all experiments, Cyantraniliprole was used at a low dose of 10 mg / kg (Low dose group – LD) or high dose 150 mg / kg (High dose group – HD), tap water was used as a vehicle. In the first experiment, males, on postnatal day (DPN) 21, were distributed into groups LD, HD or control (C), which received only vehicle. Exposure started in the DPN25, via intragastric and euthanasia occurred by anesthetic saturation in the DPN67. Sperm were collected from the vas deferens for the analysis of mitochondrial activity of the intermediate part, cholinesterase activity – ChE, lipid peroxidation – LPO, glucose-6-phosphate dehydrogenase activity - G6PD and malate dehydrogenase activity – MDH. In the second experiment, males, DPN21, were distributed into exposure groups: LD, HD and C. Treatment started at DPN25, via intragastric. Half of the animals in each group were euthanized by anesthetic saturation in the DPN67. The other animals in each group were kept alive for another 42 days, without receiving cyantraniliprole, originating the recovery groups, the euthanasia of these animals occurred by anesthetic saturation in the DPN110. Plasma was collected from all groups for determination of testosterone concentration. The testes were weighed and used for sperm count, evaluation of the antioxidant system and histological and stereological parameters. Spermatozoa from the vas deferens were used for morphological evaluation. In the third experiment, the effects of cyantraniliprole were observed in adult females, DPN67, distributed in groups C, LD and HD (n=6 per group). Euthanasia occurred in DPN95, blood was collected, for progesterone dosage, uterus and ovaries for morphometric and histopathological evaluation, ovarian follicle count and biochemical markers of oxidative stress. The measurement of the mass (g) of the liver, kidneys, pituitary and adrenal glands was performed. The progesterone concentration and the weight of the uterus, ovary, liver, kidneys, adrenal gland and a were higher in LD. The number of estrous cycles decreased and the cycle duration increased in HD animals. The glandular epithelium of the uterus was larger and the number of ovarian follicles, primordial + primary, preantral, antral and atresic was reduced in LD. Histopathological analysis revealed the occurrence of metaplasia of the luminal epithelium of the uterus with the presence of mitosis and polymorphism of the glandular epithelium cells, in addition to the presence of inflammatory infiltrate in the endometrium. In the ovaries, congested blood vessels and rete ovarii hyperplasia were observed. In utero cholinesterase activity was lower at both doses. Higher occurrence of lipoperoxidation and SOD enzyme activity occurred only in LD, and GST activity was increased in LD and HD. In the ovary, cholinesterase activity was increased in animals treated with both doses, and GPx activity was higher only with LD. As a result, we observed that in the first experiment,

mitochondrial and MDH activity decreased in sperm exposed to LD, without increasing lipid peroxidation. The activities of G6PDH and ChE were similar between groups. In the second experiment the antioxidant system activity between exposure and recovery groups was similar, however in vitro testosterone production increased in LD and HD and decreased in LDR and HDR. The number of Sertoli cells was lower in all groups that received cyantraniliprole in both the exposure and recovery phases. Phase I-V of the seminiferous epithelium cycle was increased and phase VII-VIII, decreased in the groups that received the treatment in both periods. The proportion of lumen and stroma was greater, and the epithelium smaller, only in LD, however the number of sperm per gram of testis and daily sperm production was decreased in animals that received both doses of cyantraniliprole, regardless of the phase of the testis. experiment. As a consequence, the number of normal sperm was lower in the groups that received Cyantraniliprole compared to the control. The main morphological abnormalities observed in sperm were: lack of curvature and isolated heads, broken and curled tails, and lack of acrosome integrity. In the third experiment, the progesterone concentration and the weight of the uterus, ovary, liver, kidneys, adrenal gland were higher in LD. The number of estrous cycles decreased and the cycle duration increased in HD animals. The glandular epithelium of the uterus was larger and the number of ovarian follicles, primordial + primary, preantral, antral and atresic was reduced in LD. Histopathological analysis revealed the occurrence of metaplasia of the luminal epithelium of the uterus with the presence of mitosis and polymorphism of the glandular epithelium cells, in addition to the presence of inflammatory infiltrate in the endometrium. In the ovaries, congested blood vessels and rete ovarii hyperplasia were observed. In utero cholinesterase activity was lower at both doses. Higher occurrence of lipoperoxidation and SOD enzyme activity occurred only in LD, and GST activity was increased in LD and HD. In the ovary, ChE was higher in animals treated with both doses, and GPx activity was higher only with LD. These results show that the low dose of cyantraniliprole impaired the cell membrane and sperm energy production and that the damage caused by exposure to cyantraniliprole during puberty was maintained or accentuated, even after the recovery period, ie, in adulthood. The female genital tract has also undergone changes, which may represent impairment of fertility for these individuals.

Key words: insecticide; oxidative stress; puberty; reproduction.

LISTA DE ABREVIATURAS E SIGLAS

BHT	Barreira hematotesticular
Ca2+	Íon cálcio
CaRs	Receptores sensíveis ao Ca2+
ChE	Colinesterase
DL50	Dose letal capaz de matar 50% de uma população teste
DPN	Dias pós-natal
EGF	Fator de crescimento epidérmico
EO	Estresse oxidativo
ERO	Espécies reativas de oxigênio
FGF	Fator de crescimento de fibroblasto
FSH	Hormônio folículo estimulante
G6PD	Glicose-6-fosfato
GnRH	Hormônio liberador de gonadotrofinas
GPX	Glutationa peroxidase
GST	Glutationa S transferase
IGF-I	Fator de crescimento semelhante a insulina I
LH	Hormônio luteinizante
LOAEL	Menor dose onde se observa efeito adverso ou tóxico
LPO	Lipoperoxidação
MAPA	Ministério da Agricultura Pecuária e Abastecimento
MDH	Malato desidrogenase
NAADP	Ácido nicotínico adenina fosfato dinucleotídeo
Ry	Rianodina
RyR	Receptor de rianodina
SOD	Superóxido dismutase
T	Testosterona
TGFα	Fator de crescimento transformador α
TGFβ	Fator de crescimento transformador β
WHO	World Health Organization

SUMÁRIO

1 INTRODUÇÃO

1.1 AGROTÓXICOS E SAÚDE HUMANA

Com o constante crescimento populacional mundial, a produção agrícola necessitou evoluir e tornar-se cada vez mais eficiente. Essa é a principal justificativa para a produção em larga escala de agrotóxicos, que se iniciou após as guerras mundiais pelas indústrias químicas, até então, produtoras dos venenos utilizados como armas químicas. Essas encontraram na agricultura, principalmente de países subdesenvolvidos, um novo mercado consumidor (LONDRES, 2011).

Os agrotóxicos, também conhecidos como herbicidas, pesticidas, inseticidas e até mesmo por veneno, são substâncias utilizadas na agricultura, tendo por objetivo o controle de pragas e por consequência o aumento da produtividade. Apresentam grande variabilidade em sua composição, sendo, agentes químicos, naturais ou sintéticos, com função de interferir no metabolismo, ocasionando danos tanto a microrganismos como a vegetais, e a animais invertebrados e vertebrados (ARAÚJO et al., 2007). Devido à grande utilização e ao alto poder de dispersão, independente do modo de aplicação, os agrotóxicos podem ser detectados no solo, na água e no ar, estando presentes em todos os ambientes e ecossistemas, além disso, apresentam propriedades de bioacumulação ao longo de toda a cadeia trófica, sendo, invariavelmente os seres humanos os receptores finais (BLAIR et al., 2005).

O Brasil consome 1 milhão de toneladas de agrotóxicos por ano, equivalendo a 5,2 Kg de veneno agrícola por habitante (LONDRES, 2011), e apesar da subnotificação, casos de intoxicação são frequentemente relatados. As intoxicações agudas por agrotóxicos afetam, principalmente, pessoas expostas em seu ambiente de trabalho enquanto as intoxicações crônicas podem afetar toda a população, pois são decorrentes da exposição múltipla aos agrotóxicos, geralmente em baixas doses. Dentre os efeitos associados à exposição crônica a ingredientes ativos de agrotóxicos podem ser citados infertilidade, impotência, abortos, malformações, neurotoxicidade, desregulação hormonal, efeitos sobre o sistema imune e câncer (BRASIL. MINISTÉRIO DA SAÚDE, 2015).

Pesquisas que buscam novas moléculas agroquímicas com efeitos menos drásticos a saúde dos ecossistemas são incessantes, o custo é estimado em aproximadamente 256 milhões de dólares e são realizadas principalmente por empresas de desenvolvimento (SILVA; GRIGOLLI, 2016). Dentre as novas moléculas pode-se destacar o grupo das diamidas antranílicas, como cyantraniliprole, que recentemente foi registrada no Ministério da Agricultura Pecuária e Abastecimento (MAPA). Tem classificação toxicológica IV, pouco tóxica, e classificação do potencial de periculosidade ambiental III, ou seja, perigoso ao meio ambiente. É formulado para dispersão em óleo ou suspensão concentrada em óleo, e pode ser utilizado em aplicações terrestres e aéreas, segundo as especificações do fabricante (DUPONT DO BRASIL S/A, 2016).

O cyantraniliprole é um inseticida sistêmico, com ação por contato e ingestão, que atua modulando os canais de cálcio (Ca^{2+}) conhecidos receptores de rianodina (RyR) dos insetos (PINTO, 2020). Mamíferos expressam três isoformas desses receptores: RyR1 e RyR2, distribuídos predominantemente no músculo esquelético e cardíaco, respectivamente, e RyR3 heterogeneamente distribuídos. Os insetos, ao contrário, expressam uma única forma de receptor, compartilhando 47% de homologia com RyRs de mamíferos (TAKESHIMA et al., 1994).

A DL_{50} (dose letal capaz de matar 50% de uma população teste) do cyantraniliprole, por via oral, informada na bula de distribuição foi estabelecida em ratos fêmeas como superior a 5000 mg/kg, no entanto, não há registro da LOAEL (menor dose onde se observa efeito adverso ou tóxico) (ANEXO C). Neste sentido esta pesquisa teve como objetivo verificar os possíveis efeitos da administração oral do cyantraniliprole sobre órgãos do trato genital masculino na idade juvenil e trato genial feminino de ratos Wistar adultos.

1.2 Função reprodutora em machos

A função reprodutora masculina está sob o controle da rede neuro-hormonal que é operada pelo eixo hipotálamo–hipófise-testículo ou eixo reprodutivo (figura 1.A) (SHARPE, 1994). Neste eixo funcional, o hipotálamo produz o hormônio liberador de gonadotrofina (GnRH) que controla a liberação, pela hipófise, do hormônio luteinizante (LH) e do hormônio folículo-estimulante

(FSH) que atuam no espaço intersticial e nos túbulos seminíferos contorcidos do testículo, respectivamente (KAKAR; MALIK; MAZHAWIDZA, 2004).

O LH regula a atividade esteroidogênica de células de Leydig para a produção, principalmente, de testosterona (T) e de 17β-estradiol no interstício testicular (CARREAU; WOLCZYNSKI; GALERAUD-DENIS, 2010). O FSH regula a função das células de Sertoli (GRISWOLD, 1998). A célula de Sertoli está presente nos túbulos seminíferos e se estende da lâmina basal até a luz do túbulo seminífero. Tem como principal função o apoio estrutural para as células da linhagem germinativa e a formação da BHT através de junções oclusivas. Essas oclusões organizam o epitélio em ambiente basal (onde localizam-se as espermatogônias e os espermatócitos I em pré-leptóteno) e ambiente adluminal (onde localizam-se os espermatócitos I em zigóteno, espermatócitos II e espermátides em diferentes estágios de diferenciação). O ambiente adluminal proporciona um meio isolado do sistema imunológico, sendo de extrema importância para que o processo da espermatogênese ocorra de forma correta (FRANÇA et al., 2016).

As células de Sertoli secretam nutrientes (aminoácidos, carboidratos, lipídios, vitaminas e íons metálicos), fatores de crescimento (fator de células-tronco, fatores de crescimento transformadores alfa (TGF-α) e beta e (TGF-β)), fator de crescimento semelhante a insulina-I (IGF-I), fator de crescimento de fibroblastos (FGF) e fator de crescimento epidérmico (EGF) (MRUK; CHENG, 2004) e fatores necessários para o metabolismo das células germinativas (lactato, transferina e proteína de ligação a andrógenos) (SKINNER, 2005). Também está envolvida na transdução dos sinais provenientes do hormônio folículo estimulante (FSH) e da testosterona em fatores que são essenciais para espermatogênese (SMITH; WALKER, 2015), exerce participação ativa no processo de espermiação, realizam a fagocitose dos corpos residuais, secretaram fluídos para o lúmen do túbulo seminífero para facilitar a migração dos espermatozoides para o epidídimo (FOLEY, 2001; GRISWOLD, 1998). Dessa forma, alterações no número, estrutura e função deste tipo celular podem resultar no comprometimento da espermatogênese e má formação dos espermatozoides (BOEKELHEIDE; JOHNSON; RICHBURG, 2005).

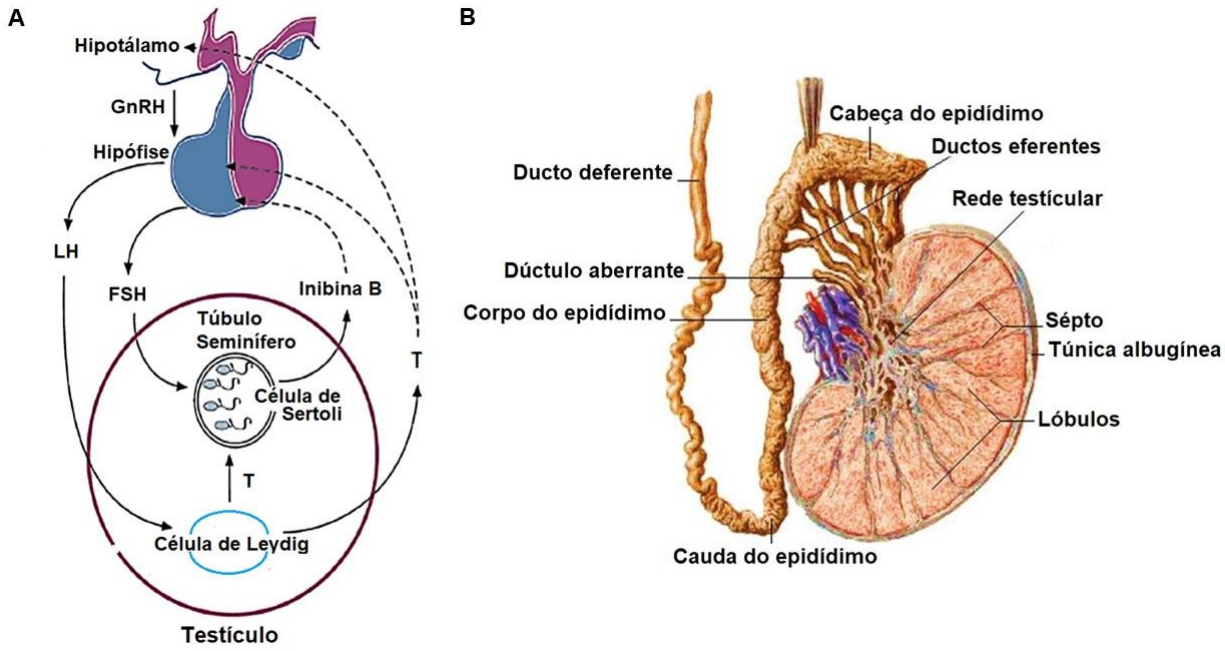


Figura 1. Eixo hormonal e anatomia do sistema reprodutor masculino. **A**, Eixo hipotalâmico – hipofisário – testicular. Linhas contínuas representam secreção de hormônios; Linhas tracejadas indicam controle retrocontrole negativo. GnRH - hormônio liberador de gonadotrofinas; LH – hormônio luteinizante; FSH – hormônio folículo estimulante; T – testosterona. Fonte: Aires, 2018. **B**, Corte sagital mediano do testículo mostrando as invaginações da túnica albugínea formando os séptulos que subdividem o órgão em lóbulos, e estes abrigam a linhagem espermatogênica. Essas estruturas convergem para a região central do órgão para formar a rede testicular e posteriormente os ductos eferentes, por onde os espermatozoides alcançam o epidídimo, um túbulo único, altamente enovelado, subdividido em cabeça, corpo e cauda do epidídimo. Fonte: Netter, F. H, 2020.

Existem quatro principais eventos que caracterizam a espermatogênese: (A) renovação celular pela mitose, (B) amplificação das espermatogônias por mitose e diferenciação, (C) redução do número de cromossomos por meiose, e (d) a transformação de uma célula convencional em célula complexa e altamente diferenciada, o espermatozoide, pelo processo metamórfico denominado espermiogênese (KERR et al., 2006). A duração total da espermatogênese em mamíferos, baseada em 4,5 ciclos espermatogênicos, é de aproximadamente 30 a 75 dias (SHARPE, 1994), sendo geralmente constante dentro de cada espécie

(FRANÇA; AVELAR; ALMEIDA, 2005). Cada geração de células germinativas se encontra em um mesmo estágio de desenvolvimento, sendo produzidas aproximadamente ao mesmo tempo e de maneira sincronizada. As várias gerações destas células formam associações celulares de composição fixa, denominadas estágios do ciclo da espermatogênese. No rato, o número de estágios da espermatogênese são 14 (Figura 2), mas este número varia de acordo com a espécie (LEBLOND; CLERMONT, 1952). Para que a espermatogênese ocorra de forma correta são necessárias interações entre as células da linhagem germinativa e os componentes somáticos do testículo, sendo que, uma alteração em qualquer fase da espermatogênese pode levar à interrupção do funcionamento correto do tecido e culminar na infertilidade (BREMNER et al., 1994).

Os espermatozoides produzidos são transportados do testículo, como células imóveis e imaturas, e no epidídimo passam por processos de maturação para adquirir a capacidade potencial de movimentar-se para frente e fertilizar oócitos (ROBAIRE; HINTON; ORGEBINCRIST, 2006).

O epidídimo (figura 1.B) é um ducto único, altamente enovelado, comumente dividido nas regiões: segmento inicial, cabeça, corpo e cauda, diferenciadas pelas características da morfologia epitelial, ao longo de seu comprimento e, também, pela composição do meio luminal, como resultado de secreção ativa e absorção de água, íons, solutos orgânicos, proteínas, glicoproteínas e glicolipídios pelas células epiteliais (ROBAIRE; HINTON; ORGEBINCRIST, 2006). O epidídimo desempenha importante papel na proteção dos espermatozoides tal como ocorre na blindagem ao estresse oxidativo (EO), ao secretar substâncias antioxidantes e remover espécies reativas de oxigênio (EROs). Em termos funcionais, este ducto está envolvido não só na maturação, como também no transporte, concentração, proteção e armazenamento dos espermatozoides (ZUBKOVA; ROBAIRE, 2004).

A maturação espermática no epidídimo parece depender de uma interação altamente regulada entre a lâmina própria, células epiteliais e fluído luminal que banha os espermatozoides (ROBAIRE; HINTON; ORGEBINCRIST, 2006). Este processo consiste em um intenso remodelamento da membrana do espermatozoide, em que proteínas de origem testicular são removidas ou modificadas e proteínas epididimárias são secretadas e adsorvidas à membrana

espermática ou apenas interagem com o gameta, a fim de que este adquira sua capacidade funcional (KRAPF et al., 2012). Cada etapa da maturação é decisiva para a qualidade espermática final, e é dependente da ação de andrógenos (DACHEUX; DACHEUX, 2014).

A maturação espermática no epidídimo parece depender de uma interação altamente regulada entre a lâmina própria, células epiteliais e fluído luminal que banha os espermatozoides (ROBAIRE; HINTON; ORGEBINCRIST, 2006). Este processo consiste em um intenso remodelamento da membrana do espermatozoide, em que proteínas de origem testicular são removidas ou modificadas e proteínas epididimárias são secretadas e adsorvidas à membrana

espermática ou apenas interagem com o gameta, a fim de que este adquira sua capacidade funcional (KRAPF et al., 2012). Cada etapa da maturação é decisiva para a qualidade espermática final, e é dependente da ação de andrógenos (DACHEUX; DACHEUX, 2014).

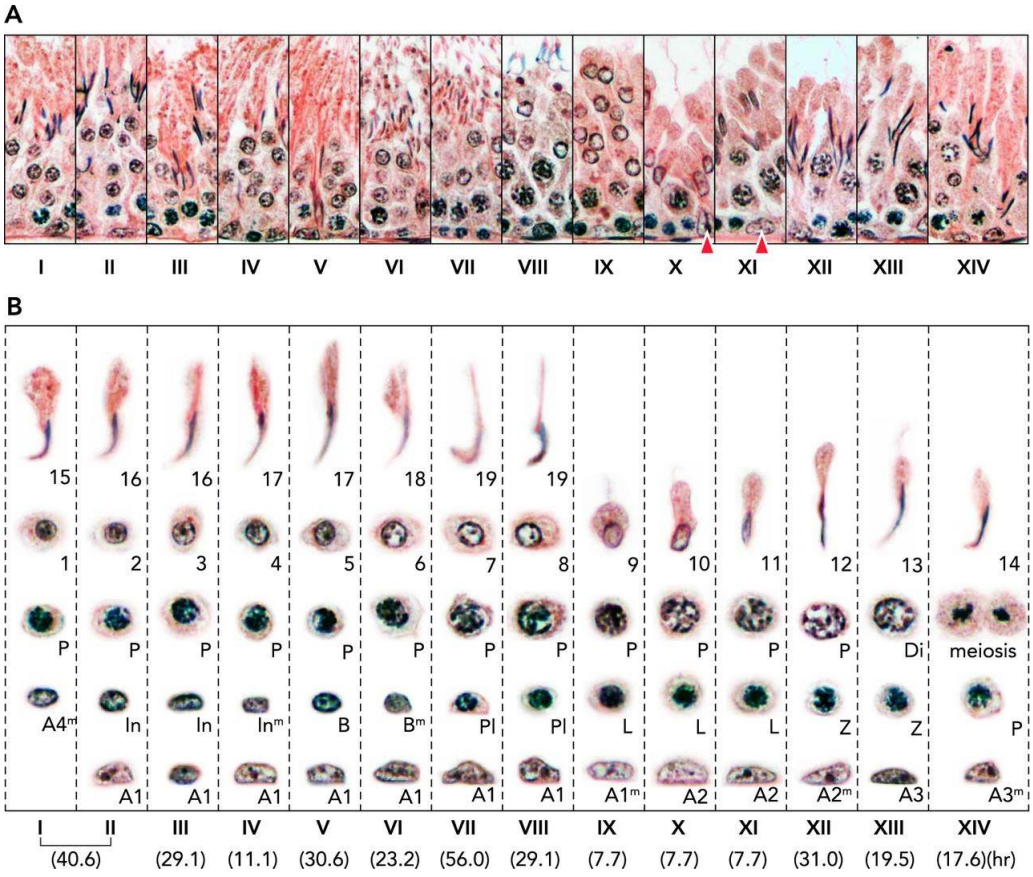


Figura 2. Ciclo do epitélio seminífero da espermatogênese. (A) Cada fase do ciclo do epitélio seminífero ilustra a associação única de células germinativas

específicas com a célula de Sertoli (ponta de seta vermelha). (B) Mostra os diferentes tipos de células germinativas que são encontrados em cada fase do ciclo. Por exemplo, na fase VIII, com duração de aproximadamente 29,1 horas no rato, espermátides 19 alinham-se próximo ao lúmen tubular para a espermiação. Todo o ciclo de I a XIV leva aproximadamente 12,9 dias para se completar. Porém, para que uma espermatogônia A tipo II se torne uma espermátide 19, são necessários cerca de 4,5 ciclos, o que leva por volta de 58 dias (adaptado de Xiao et al. (2014).

1.3 DESENVOLVIMENTO PÓS-NATAL DO SISTEMA GENITAL EM RATOS MACHOS

Segundo Ojeda *et al.*, (1980), o desenvolvimento do rato macho ocorre em quatro fases: neonatal (dias pós-natal – DPN 1 - 7), infantil (DPN 8 – 21), juvenil (DPN 22 – 35) e puberal (DPN 36 – 55 ou 65). No período puberal ocorre o rápido crescimento testicular, mudanças de secreção de LH, aumento gradual de testosterona sérica e início da espermatogênese (ROBB; AMANN; KILLIAN, 1978).

A produção de testosterona se inicia no final do período gestacional e diminui logo após o nascimento. Nas fases infantil e juvenil (DPN 8 – 35) os andrógenos primários são produzidos, e esses incluem a androstenediona, 5-α-androstanediol e diidrotetosterona (PODESTÁ; RIVAROLA, 1974). As células de Leydig imaturas iniciam o seu processo de diferenciação em células adultas entre o DPN 28 – 56 apresentando baixa atividade mitótica, mas alta síntese de testosterona (BENTON; SHAN; HARDY, 1995).

A puberdade ocorre em decorrência de uma cascata de eventos que levam a maturação do eixo hipotálamo-hipófise-testículo. Neste período, o aumento da pulsatilidade do hormônio GnRH induz o aumento da circulação de LH e FSH, resultando na mudança de perfil hormonal característico da puberdade. Esses eventos desencadeiam a síntese e secreção de esteroides, como a testosterona, marcando o início do ciclo reprodutivo da espécie (OJEDA; SKINNER, 2006). Um sinal da instalação da puberdade em machos é a separação prepucial (KORENBROT; HUHTANIEMI; WEINER, 1977). No entanto, as espermátides maduras são encontradas nos testículos de ratos no DPN 40 e nos epidídimos, a presença de espermatozoides é notada no DPN 50. A máxima produção de espermatozoide se dá no DPN 75, e a maior

concentração de espermatozoides armazenados no epidídimo ocorre no DPN 100 (ROBB; AMANN; KILLIAN, 1978).

1.4 FUNÇÃO REPRODUTORA EM FÊMEAS

Nos ovários das fêmeas, o ciclo ovulatório inclui múltiplos eventos relacionados a foliculogênese, ovulação e preparação do sistema genital para a fertilização e implantação que culminam na gestação (BOUÉ; BOUÉ; LAZAR, 1975). A ovulação resulta de interações entre regiões hipotalâmicas, hipófise e ovário. O hipotálamo libera GnRH e como resposta, a hipófise é estimulada e os níveis de FSH e LH aumentam subitamente. Este surto ovulatório das gonadotrofinas ocasiona o desenvolvimento folicular e estimula o ovócito primário do folículo maduro a terminar a primeira divisão meiótica, além de fornecerem estímulos para a cascata de eventos ovarianos que resulta na ovocitação do ovócito secundário em metáfase da segunda divisão meiótica (BOUÉ; BOUÉ; LAZAR, 1975).

A ovulação de um ovócito II fertilizável requer o crescimento, maturação e diferenciação da célula germinativa, das células da granulosa, das células endócrinas da teca e a formação de corpo lúteo. Todos estes eventos são susceptíveis a efeitos tóxicos, como por exemplo, as divisões das células da granulosa durante o crescimento folicular e a diferenciação das células da granulosa e da teca (TILLY, 1997). Após a ovulação, as células foliculares se diferenciam em corpo lúteo e iniciam a secreção de progesterona. Nem todos os folículos que são estimulados a crescer serão ovulados; a maioria dos folículos degenera por um processo denominado atresia e diferenciam-se posteriormente em interstício (LEVINE, 2015).

Na idade adulta, o útero de ratas apresenta a luz dos cornos uterinos completamente separados e eles se abrem na luz da vagina como orifícios externos pareados. A parede uterina é composta pela mucosa (endométrio), duas camadas de músculo liso (miométrio) e camada adventícia. Uma camada de células epiteliais colunares forma as glândulas uterinas que se projetam para o endométrio (figura. 3) (WESTWOOD, 2008). A rápida e sincrônica formação das glândulas uterinas podem ser alteradas pela administração de compostos exógenos de uma maneira tempo e dose-específica (DIXON et al., 2018).

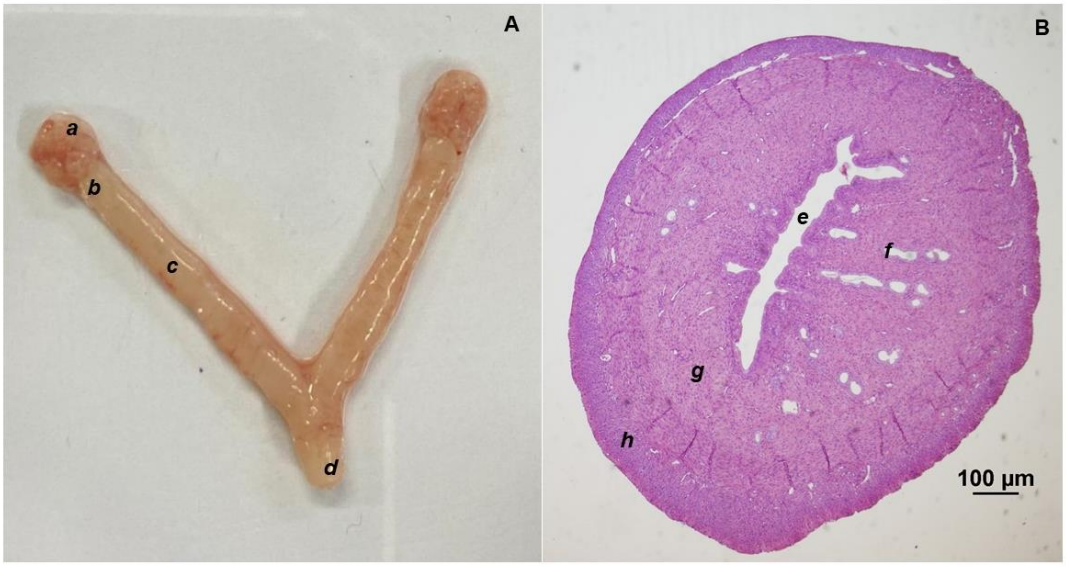


Figura 3. Órgãos do trato genital de ratos fêmeas. Em A, vista anterior dos órgão genitais de ratos fêmeas. ***a***, ovário; ***b***, tuba uterina; ***c***, corno uterino; ***d***, vagina. Em B, corte transversal do corno uterino. ***e***, luz uterina; ***f***, glândula endometrial; ***g***, endométrio; ***h***, miométrio. H&E. Fonte: Arquivo próprio.

Durante o ciclo estral, o útero de uma rata sexualmente madura passa pelas seguintes modificações: no proestro, o lúmen é distendido com fluido, as células epiteliais são cuboides ao invés de colunares e o estroma e o miométrio demonstram uma intensa infiltração de leucócitos; no estro o endométrio encontra-se hiperêmico, o lúmen está distendido ao máximo e a infiltração de leucócitos persiste; no metaestro a quantidade de fluído uterino está diminuída, as células do epitélio cuboide apresentam degeneração vacuolar, e a infiltração de leucócitos no estroma e no miométrio diminui; no diestro o epitélio é regenerado e a infiltração leucocitária é diminuída ao mínimo (DIXON et al., 2014). O exame microscópico do lavado vaginal é o método mais adequado para determinar a fase do ciclo estral e é utilizado em toxicologia para avaliar os possíveis efeitos tóxicos dos agentes químicos sobre a ciclicidade do estro (DIXON et al., 2018).

2 JUSTIFICATIVA

O Brasil está entre os maiores consumidores de agrotóxicos do mundo e pelo impacto social e ambiental causado pelo uso desordenado, tornam-se cada vez mais relevantes estudos que identifiquem os efeitos negativos dessas substâncias sobre a saúde humana. Os riscos da exposição não se limitam ao homem do campo, atingem mananciais, o solo, o ar, e os animais. Além disso, a maioria dos alimentos comercializados nas cidades apresentam resíduos de agrotóxicos, expondo essa população a pequenas doses, porém de forma crônica. Os casos de intoxicação, seja aguda ou crônica, apresentam grande subnotificação e descentralização de informações, decorrentes de fatores diversos, como dificuldade de acesso dos agricultores às unidades de saúde, inexistência de centros de saúde em regiões produtoras importantes, dificuldade de diagnóstico e de relacionar os problemas de saúde com a exposição aos agrotóxicos, escassez de laboratórios de monitoramento biológico e inexistência de biomarcadores precoces e/ou confiáveis.

Diversos estudos correlacionam o aumento da utilização dos agrotóxicos com a subfertilidade masculina e feminina no mundo, principalmente pelo crescente número de casais que apresentam infertilidade idiopática. Os relatos mostram que diversas classes de agrotóxicos, mesmo em baixas doses, podem interagir com o organismo humano mimetizando efeitos hormonais ou induzindo a ocorrência de estresse oxidativo, que exercem efeitos adversos sobre os órgãos reprodutivos e na produção de gametas. Outro fato relatado é que intoxicação com resíduos de agrotóxicos pode ocorrer desde o momento em que a introdução alimentar é iniciada, através do leite materno e da água, e por outros alimentos contaminados ao longo da vida. Assim os humanos estão sujeitos a exposição a essas substâncias em diversos períodos críticos do desenvolvimento, como na puberdade, onde os eventos relacionados a maturação do eixo hipotálamo - hipófise - gônada, produção e liberação dos gametas são iniciadas.

Substâncias que perturbem essa fase de desenvolvimento, podem ter efeitos que serão observados instantaneamente ou na vida adulta. Até o momento não existem relatos sobre o efeito do cyantraniliprole sobre os aspectos da reprodução em humanos, mas sabe-se que no Brasil seu uso é

indicado para 23 tipos de cultura, incluindo desde a soja até o pimentão. Levando em consideração o potencial de uso dessa substância, o presente estudo tem grande aplicabilidade ao avaliar os efeitos da exposição ao cyantraniliprole em diferentes doses sobre o sistema genital masculino de ratos púberes e adultos, e sobre o sistema genital feminino em ratas adultas. Os resultados obtidos podem contribuir para compreensão dos efeitos do cyantraniliprole em mamíferos, bem como para medidas educativas para o uso racional dessa substância.

3 OBJETIVOS

3.1 Gerais

Entendendo a relevância clínica, social e política vinculada ao uso dos agentes químicos utilizados na agricultura e pela falta de informações da ação do cyantraniliprole sobre o trato genital feminino e masculino, foram destacados os seguintes objetivos: (i) avaliar se a exposição ao cyantraniliprole durante o período puberal de ratos exerce efeito tóxico sobre o sistema reprodutor masculino na idade púbere (avaliação imediata) e adulta (após período de recuperação); (ii) avaliar os efeitos da exposição ao cyantraniliprole sobre sistema reprodutor feminino de ratas *Wistar* ; (iii) contribuir com dados científicos que norteiem novas pesquisas, políticas educativas e de saúde pública que promovam o uso adequado do cyantraniliprole.

3.2 Específicos

Em machos:

- Avaliar os efeitos da exposição oral ao cyantraniliprole, nas doses de 10 e 150 mg/kg durante o período peripuberal, na idade púbere (e após período de recuperação (sem exposição) e adulta.
- Determinar a influência do cyantraniliprole sobre a função testicular por meio da avaliação de parâmetros espermáticos e sobre os morfologia tecidual por meio de avaliação dos aspectos histopatológicos
- Analisar a fisiologia endócrina testicular através da dosagem de testosterona plasmática, intratesticular e produção de testosterona in vitro.
- Identificar se os marcadores de sistema oxidativo, nos testículos e espermatozoides, são alterados pela exposição ao inseticida.
- Avaliar a qualidade espermática por meio das avaliações do metabolismo celular e morfologia espermática.

Em fêmeas:

- Avaliar os efeitos da exposição oral cyantraniliprole, nas doses de 10 e 150 mg/kg, sobre o sistema reprodutor feminino de ratas adultas.

- Determinar a influência do cyantraniliprole sobre aspectos histopatológicos do útero, ovário e sobre a concentração plasmática de progesterona.

- Avaliar se os marcadores de sistema oxidativo, nos ovários e uteros, são alterados pela exposição ao inseticida.

4 ARTIGOS

Este estudo foi desenvolvido no Laboratório de Toxicologia e Distúrbios Metabólicos da Reprodução, Universidade Estadual de Londrina – UEL, Laboratório de Análises Bioquímicas, Laboratório de Biologia Estrutural e Funcional e Laboratório Experimental de Morfologia da Universidade Estadual do Oeste do Paraná – Unioeste.

ARTIGO 1

CYANTRANILIPROLE ALTERS THE SPERM ENERGY PRODUCTION BY OXIDATIVE DAMAGE IN PERIPUBERAL RATS WISTAR

Artigo será submetido à revista — “Food and Chemical Toxicology”
ISSN: 0278 - 6915;
F.I. 2019: 4.6
Qualis CAPES 2013 - 2016 (Medicina II): A.2

CYANTRANILIPROLE ALTERS THE SPERM ENERGY PRODUCTION BY OXIDATIVE DAMAGE IN PERIPUBERAL RATS WISTAR

Suellen Ribeiro da Silva Scarton[1,2*], Felipe Tsuzuki[1], Dayane Priscila dos Santos[1,2], Fernando Antônio Briere[3], Ana Tereza Bittencourt Guimarães[3], Célia Cristina Leme Beu[3], Glaura Scantamburlo Alves Fernades[1]

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

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

3 - Medical and Pharmaceutical Sciences Center, State University of Western Paraná - UNIOESTE, Universitária street, 1619, PR, Postal code: 85819-110, Cascavel, Paraná, Brazil

* Corresponding author

Email adress: suellen.scarton@uel.br

Abstract

Cyantraniliprole is a synthetic insecticide that modulates Ca^{2+} receptors of the ryanodine type (RyR). It is known that substances used for pest control can interact with mammalian systems facilitating the occurrence of oxidative damage, especially in critical periods of development. This study aimed to assess whether intragastric exposure to Cyantraniliprole impairs sperm quality during peripubertal period. For this, male Wistar rats (postnatal days (DPN) 21) were distributed in three experimental groups: Control, 10 or 150 mg/kg b.w and submitted to the experimental design from DPN 25 to the DPN 66; euthanasia occurred at DPN 67. Sperm from the tail of the epididymis or the deferent vas were used in the analysis of mitochondrial activity, cholinesterase activity - ChE, lipid peroxidation - LPO, glucose - 6 - phosphate dehydrogenase - G6PDH activity, and malate dehydrogenase - MDH activity. Mitochondrial and MDH activity were decreased in the spermatozoa exposed to low dose, however this dose caused increased lipid peroxidation. G6PDH and ChE activity were similar between experimental groups. These results show that the lowest dose of cyantraniliprole damages cell membranes and decreased energy production capacity of the sperm.

Keywords: Insecticide, puberty, sperm, mitochondria.

Introduction

Insecticides are substances used to control pests of agricultural importance. They have great variability of chemical, synthetic, or natural compounds (Araújo et al., 2007). Regardless of the mode of application, they are detected in the soil, in water, and the air due bioaccumulation properties along the food chain, with the human being the final receptors (Blair et al., 2005). Cyantraniliprole is a synthetic insecticide, derived from the *Ryania speciosa* Vahl. (Salicaceae: Malpighiales) plant, belonging to the class of anthranilic diamides (Lahm et al., 2005), which regulates the release of intracellular Ca^{2+} ($[Ca^{2+}]i$) through Ca^{2+} channels of the ryanodine type (RyR) (Cordova et al., 2006). The sperm from rats (Jimenez-Gonzalez et al., 2006) and humans (Costello et al., 2009; Harper et al., 2004) present varying amounts of RyRs, mainly in the connection piece, which connects the head to the piece intermediate. In this region, the redundant nuclear envelope (RNE) functions as an intracellular Ca^{2+} stock, which proves the $[Ca^{2+}]i$ necessary to initiate and regulate axonemal hyperactivation (Bedu-Addo et al., 2008; Ho and Suarez, 2003; Mendoza et al., 2012).

It is known that Ca^{2+} (Bertero and Maack, 2018; Staldoni de Oliveira et al., 2020) and several classes of insecticides (Abdollahi et al., 2004; Jabłońska-Trypuć et al., 2017) can modulate the occurrence of stress oxidative (EO), that recognized as an important cause of male infertility by decreasing the quality of sperm parameters (Agarwal et al., 2003; Kao et al., 2008; Ko et al., 2014; Tremellen, 2008). Sperm, like other aerobic cells, perform a constant balance between the production of reactive oxygen species (ROS) and antioxidation (SIES, 1993). Physiological levels of ROS maintain the normal functioning of the

cell, however the increase in its production can impair cell function and survival (De Lamirande and Gagnon, 1995). ROS generation by sperm can occur in two ways: (I) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the plasma membrane level (Aitken et al., 1992) and (II) level-dependent NADPH-oxide reductase mitochondrial (Gavella and Lipovac, 1992).

The occurrence of OS in the juvenile and peripubertal periods (postnatal days (PND) 22-65) is potentially toxic to germ cells since these periods represent the window of vulnerability for the postnatal development of the male reproductive system (Golub et al., 2008). In a previous study, our research group describes sperm changes and the occurrence of OS in male rats exposed to toxins during these periods (R. Erthal et al., 2020; R. P. Erthal et al., 2020; Ogo et al., 2018, 2017). In rats, it is known that during the periods of childhood and youth (PND 8-35), primary androgens are produced (Podestá and Rivarola, 1974), and between PND 28 and 56 Leydig cells are differentiated (Benton et al., 1995). In the peripubertal period (PND 35-65), the hypothalamic-pituitary-testis axis matures and Leydig cells start producing testosterone (Golub et al., 2008). The disturbance of these processes causes damage to spermatogenesis and steroidogenesis, and these can persist in the period of sexual maturity, that is, in adult life (Slimen et al., 2014). In a previous study, our research group describes sperm changes and the occurrence of OS in male rats exposed to toxins during these periods (R. Erthal et al., 2020; R. P. Erthal et al., 2020; Ogo et al., 2018, 2017). No studies were found on cyantraniliprole exposure during postnatal development period of the male reproductive system in morphological, physiological or oxidative parameters of the sperm rat. Therefore, this study

aimed to evaluate the effect of exposure to Cyantraniliprole during the peripubertal periods on the sperm of Wistar rats during.

Material and methods

Animals and experimental conditions

Male Wistar rats, DPN 21, were obtained by Central Bioterium of the State University of Western Paraná – UNIOESTE, and were acclimated to the new environment at the Laboratory of Toxicology and Metabolic Dysfunction of Reproduction of the State University of Londrina - UEL, in polypropylene boxes (43 x 30 x15) (4 animals per box) under 12 h light / dark cycle, room temperature ~ 23 °C, with free access to water and standard feed. Body weight, feed and water consumption were measured twice a week. Animal care and handling procedures were in accordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised in 1978) and with the approval of the Ethics in Use Committee of Animals of the State University of Londrina (OF. CIRC. CEUA nº134 / 2017, process n° 21106.2017.24).

Experimental design

The animals (21 days of age) were distributed in the following experimental groups (n = 6 animals/group): control group (C, which received tap water - vehicle), and two groups exposed to 3-bromo-1-(3-chloro-2-pyridyl)-4′-cyano-2′-methyl-6′-(methylcarbamoyl) pyrazole-5-carboxanilide - Cyantranilipore (Benevia® 10%m/v - Du Pont Brasil S.A., Barueri, SP, Brazil) at dose of 10 mg/kg (low dose - LD) or to 150 mg/kg (high dose - HD). Once that, nowadays there is

no knowledge on the reproductive toxicity of this chemical available in the literature, the doses used in this present experiment were based on the Safety Data Sheet information. The DL_{50} reported, orally, is > 5000 mg/kg (Safety Data Sheet, 2016).

The animals were treated orally (gavage) from DPN 25 to the DPN 66 and euthanized at DPN 67 by anesthetic saturation with Isoforine® 1.28% (Cristália, Itapira, SP, BR) on DPN 67. According to Ojeda *et al.*, (1980) this days correspond to the juvenile and peripubertal periods in male rats.

Preparation of Cyantraniliprole

Cyantraniliprole (3-bromo-1- (3-chloro-2-pyridinyl) -N- [4-cyano-2-methyl-6 - [(methylamino) carbonyl] phenyl] -1 H-pyrazole-5-carboxamide; 10 , 0% w / v), was received as a donation from the Center for Agricultural Sciences of the State University of Londrina - UEL. Tap water was used as a vehicle for the dilution of the toxic agent.

Sperm collection

The sperm from the tail of the epididymis were collected and used in the analysis of the mitochondrial activity of the intermediate piece. Biochemical analyzes of cholinesterase activity (ChE), lipoperoxidation (LPO), the activity of glucose 6 phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH) were performed with sperm obtained from the right deferent vas.

Mitochondrial activity

The mitochondrial activity of the sperm (n = 06) was determined as described by Silva *et al.*, (2014) with adaptations. Sperm obtained from the tail of the epididymis were added in microtubes containing 1 mg / mL of 3-30-diaminobenzidine (DAB) dissolved in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.03 mM Na2HPO4, KH2PO4 1.47 mM, pH 7.4) in a 1: 3 (v / v) ratio and incubated at 37 ° C for 1 h in the dark. Smears were prepared under histological slides and fixed with 10% formaldehyde for 10 min. Two hundred cells were evaluated with a phase-contrast microscope and classified as: DAB-I (stained intermediate piece, indicating that the cells maintain a complete mitochondrial activity or little loss of mitochondrial activity, which may not lead to severe impairment of motility and capacity fertilization); DAB-II (absence of staining in the intermediate part, indicating dead cells or cells that maintain minimal energy production through oxidative phosphorylation).

Biochemical analyzes

The sperm were collected from the right deferent vas in 200 µl of phosphate saline solution, pH 7.4, and centrifuged at 9,500 g for 10 min at 4 ° C. The protein quantification of the samples was determined by the method of Bradford, (1976), using bovine serum albumin (BSA) as standard, in the sequence all samples were normalized to 1.0 mg / mL of protein. The supernatants were separated and used to determine cholinesterase activity (ChE), lipid peroxidation (LPO), glucose-6-phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH) activity.

Cholinesterase activity

ChE activity (n = 06) was performed according to the method of Ellman et al., (1961) adaptations for microplate. The objective is to measure the production of thiocholine when acetylthiocholine (ATC) is hydrolyzed, which is accomplished through the continuous reaction of the thiol with the 5, 5 'dithiobis-2-nitrobenzoic acid (DTNB) to produce the yellow anion of the Acid 5-thio-2-nitrobenzoic. The reaction was carried out in duplicate in 30 µL of solution containing 0.05 DTNB and 1.5 mM ATC. ChE activity in relation to protein concentration (mg.mL-1) was calculated using the molar extinction coefficient of DTNB (1.36mM-1.cm-1). The results were expressed in nmol.min-1. mg of protein-1.

Lipid peroxidation

The LPO (n = 06) was measured to indirectly quantify the peroxides produced. The result reflects the intensity of lipid peroxidation (Lushchak et al., 2009). Measurements were performed using the method of reactive substances to thiobarbituric acid (TBARS) with an absorbance of 535 nm (Buege and Aust, 1975) compared to the standard curve for malondialdehyde (MDA), the main by-product of cellular lipid peroxidation. To prepare the test, an aliquot of 0.33 mg.mL-1 of the sample protein was added in 6.7% trichloroacetic acid (TCA) in a final volume of 60 µL and centrifuged for 5 min at 13,860 g at 4ºC. For the measurement of substances reactive to thiobarbituric acid (TBARS), the following substances were added to the microplate: 20 µL of the supernatant and different concentrations of MDA in triplicate, and reaction medium containing 21.42 mM of thiobarbituric acid (TBA), 17,86 mM NaOH (used for TBA solubilization), 0.73 M TCA, 0.032 mM butylated hydroxytoluene (BHT) and 3% ethanol (used for BHT

solubilization) in PBS buffer. Lipid peroxidation was estimated from the MDA curve, and the results are expressed in nmol of TBARS. mg of protein-1.

Glucose 6 phosphate dehydrogenase activity

G6PDH activity (n = 06) was assessed as described by Glock & Mclean, (1953), with adaptations. The principle of the method is to measure the increase in absorbance caused by the reduction of NADP+ to NADPH, at 340 nm by G6PDH. The results were expressed μmoles of reduced NADP+ x min-1 x protein-1 mg.

Malate dehydrogenase activity

MDH (n = 06) is the final enzyme of the Krebs Cycle, whose function is the regeneration of oxaloacetate through the conversion of oxaloacetate to malate. Its activity was measured by kinetic means, using the method proposed by Childress & Somero, (1979). For this purpose, a reaction system composed of 50 mM Tris-HCl pH 7.4, 0.4 mM oxaloacetate, 20 mM MgCl2, 150 μM NADH, and deionized H_2O was carried out. As soon as the system was added to the samples, an absorbance reading was made at 340 nm, for 8 minutes at 21-second intervals. The results were expressed in oxidized NADH.min-1.mg of protein-1.

Statistical analysis

The Shapiro-Wilk test was used to verify the normal distribution of the data, and the homogeneity of the variance between the groups was assessed by the Levene test. One-way analysis of variance (ANOVA) with Tukey post hoc test or

Kruskal-Wallis non-parametric test with Dunn's post hoc test was used to compare the results between the groups treated with Cyantraniliprole and the control group. Differences were considered significant when $p < 0.05$. Data are presented in mean ± S.E.M., when parametric or median, 1 and 3 ° quartiles, when non-parametric. Statistical analyzes and graphs were performed using the IBM® SPSS® Statistics program (IBM Corp. Launched in 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Mitochondrial activity

In the evaluated sperm, mitochondrial activity DAB-I was lower in the LD group when compared to the control ($p = 0.004$), but it was statistically equal when comparing the HD x C or LD x HD groups. Consequently, to this result, the mitochondrial activity DAB-II was increased in sperm belonging to the LD group when compared to the control group ($p = 0.004$); the other groups were statistically similar to each other (Figure 1).

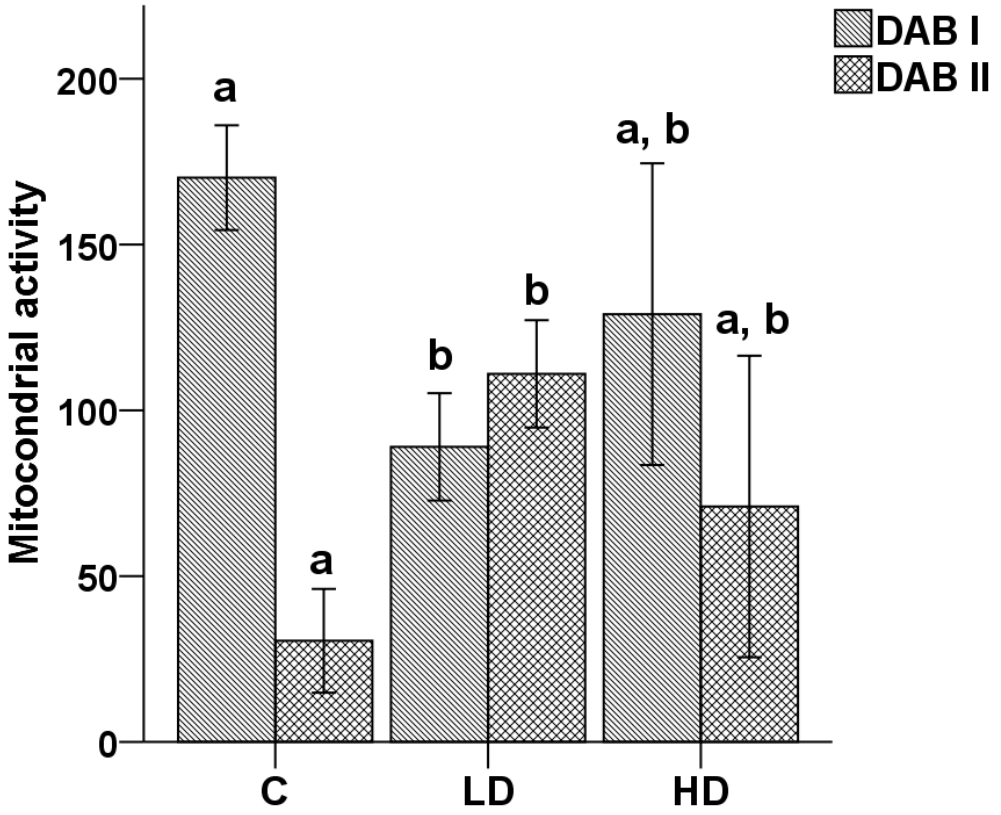


Figure 1 - Mitochondrial activity of the midle piece of sperm from animals in the control group (C), exposed to low dose 10 mg/kg (LD) or high dose 150 mg/kg (HD).

Data are presented in mean ± S.E.M., differences were considered significant when $p < 0.05$, n = 06 per group.

Cholinesterase enzyme activity - ChE

The activity of the cholinesterase enzyme in the sperm in the LD or HD groups was not statistically different from the sperm in the animals in the control group (Figure 2).

Lipid peroxidation - LPO

Exposure to the lowest dose of Cyantraniliprole caused an increase in the levels of sperm lipid peroxidation in relation to the control group ($p = 0.009$). However, the highest dose of the insecticide did not impair this parameter when compared to the control group. When comparing the effect of the lowest and highest dose, it was observed a similarly statistic between these groups (Figure 2).

Glucose 6 phosphate dehydrogenase activity – G6PDH

This analysis showed that exposure to Cyantraniliprole did not alter the activity of G6PDH in sperm, since no statistical differences were observed concerning the control group (LD x C) or (HD and C) (Figure 2).

Malate dehydrogenase activity - MDH

MDH activity in sperm was decreased after exposure to the lowest dose of Cyantraniliprole compared to the control ($p = 0.028$). Sperm from animals that received the highest dose showed increased MDH activity when compared to the group that received the lowest dose ($p = 0.011$). This parameter was similar between high dose and control groups (Figure 2).

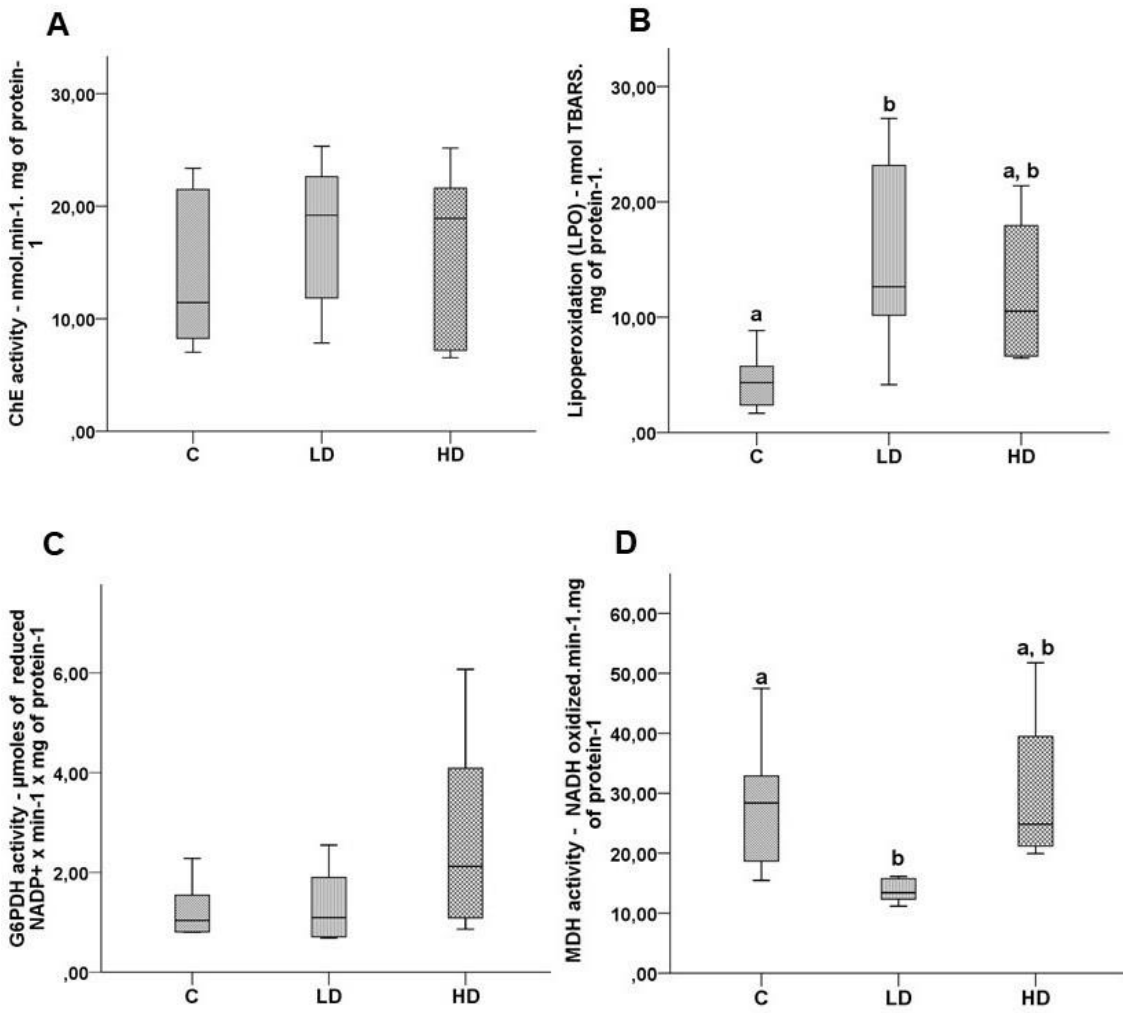


Figure 4 - Cholinesterase activity, lipoperoxidation, glucose-6-fosfato dehydrogenase activity and malate dehydrogenase activity of sperm from animals in the control group (C), exposed to low dose 10 mg/kg (LD) or high dose 150 mg/kg (HD).

Data are presented in median, 1 and 3 ° quartiles in non-parametric statistic; Differences were considered significant when $p < 0.05$, n = 06 per group.

Discussion

The current study evidenced that exposure to cyantraniliprole during peripubertal periods impair the sperm quality of rats, mainly at lowest dose used

in this work. This approach can lead to impaired fertility due to lipid peroxidation and metabolic dysfunction in the male gamete.

The action of reactive oxygen species on sperm is under fine control cellular, to be beneficial or harmful, to keep the balance between the quantities of ROS produced and the availability of eliminators (De Lamirande and Gagnon, 1995). Sperm and seminal plasma have systems capable of eliminating or neutralizing the action of ROS, which include the enzyme superoxide dismutase (SOD) (ALVAREZ et al., 1987), catalase (Jeulin et al., 1989), and the glutathione peroxidase system/reductase (Alvarez and Storey, 1989).

In the present study, the increased lipoperoxidation in sperm exposed to a lower dose of cyantraniliprole shows damage to cell membranes such as plasma membrane and membranes present in cell organelles. This harmful event can cause both cell death and loss of function. The susceptibility of sperm to oxidative stress occurs due to a large amount of polyunsaturated fatty acids present in their membranes (Tremellen, 2008), which may suffer a loss of integrity or alteration in the composition of their proteins (Aitken et al., 1995). It has been established that peroxidation of the sperm membrane leads to a decline in the potential of the mitochondrial membrane (Koppers et al., 2011), while changes in the composition of the sperm mitochondrial membrane can affect the activity of the respiratory chain and the oxidative phosphorylation pathway (R John Aitken et al., 2012; R. John Aitken et al., 2012; Amaral and Ramalho-Santos, 2010; Zhang et al., 2016). These factors culminate in the loss of energy production (AMARAL *et al.*, 2014). Therefore, in the present study, the loss of the sperm's energy capacity evidenced by the decrease in mitochondrial activity reduces cell viability and decreased sperm motility. Kao *et al.*, (2008) showed that there is a decrease

in sperm motility when there is a reduction in mitochondrial energy capacity in sperm.

In addition the sperm motility, studies have shown that sperm with greater AChE gene expression exhibit reduced motility in humans (Chavarro et al., 2010; Li et al., 2018; Zheng et al., 2017), and acetylcholine stimulates the motility of human sperm (Dwivedi and Long, 1989). However, in the current work the maintenance of the activity of ChE between experimental groups evidenced that exposed to cyantraniliprole does not change the activity of this enzyme in the sperm. It is known that ChE is found in the scourge of mobile and non-mobile sperm since its effects are related to the propagation of the flagellar wave, coordinated contractions for the flagellar beat (Chakraborty and Nelson, 1974) and also with the permeability of the spermatic membrane (Chakraborty and Nelson, 1976).

The alteration in MDH activity in sperm of the animal's exposure to cyantraniliprole showed that each of the doses acts independently on the function of this enzyme, although the enzyme glucose-6-phosphate dehydrogenase (G6PD) activity has not been altered by this insecticide. It is known that hydrogen peroxide (H_2O_2) can damaged sperm membrane and inhibit the activity of the enzyme G6PD via hexose-monophosphate; this pathway controls the availability of NADPH that will be used for energy generation by sperm (Aitken et al., 1997). Our results show that this pathway was probably not altered by the action of cyantraniliprole or by the increased peroxidation of the lipids of the sperm membranes. Cytosolic NADH cannot cross mitochondrial membranes, so its reduced cytosol equivalents (NAD+) are transferred to the mitochondria by transport systems which the MDH are fundamental for the passage of these

equivalents for use in the Krebs cycle (Minárik et al., 2002). Therefore, we can infer that the lower mitochondrial activity observed in sperm in the LD group may be related to decreased MDH activity also observed in this group. Breininger *et al.*, (2017) reported that MDH inhibitors induce decreased sperm motility during capacitation in a dose-dependent manner; however, they do not modify motility during the acrosome reaction. The role of glycolysis and oxidative phosphorylation in the Krebs cycle for the regulation of sperm motility is unclear. Studies indicate that the activity of these pathways can be species-specific and occur in different regions in sperm (Minárik et al., 2002; Mukai and Okuno, 2004; Nascimento et al., 2008; Vermouth et al., 1986).

According to the results of the present study, we showed that cyantraniliprole impairs the cellular metabolism of the male gamete of animals exposed during the peripubertal period. In general, we observed that the lowest dose of cyantraniliprole was able to induce greater damage to sperm, which differs from the classic dose-response effect, where it is possible to observe the increase in the percentage of individuals who manifest a certain effect with increased dose. This can be explained by the absorption rate of cyantraniliprole. The absorption rate for a single dose of 10 mg/kg was 60 - 83%, while for 150 mg/kg it was 31 - 40% within 48 hours (Du Pont, 2016). According these results we can infer that the adaptive capacity to chronic exposure to low doses of cyantraniliprole was inhibited. Nevertheless, these harmful events can persist into adulthood and cause infertility that can be classified as an idiopathic cause.

Conclusion

In conclusion, expose to cyantraniliprole during peripubertal period was able to induce toxicity in the sperm of pubertal rats, being more accentuated in lower dose.

Acknowledgment

The authors are grateful to CAPES (Coordinating Body for the Improvement of Postgraduate Studies in Higher Education) for providing a PhD scholarship to Scarton, S.R.S and partially financial support (Finance Code 001). This paper represents part of the PhD thesis by Scarton, S.R.S. (State University of Londrina - Brazil) under the supervision of GSA Fernandes.

Conflicts of interest

The authors declare no conflicts of interest.

References

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., Rezaie, A., 2004. Pesticides and oxidative stress: A review. Med. Sci. Monit. 10, 141–148.

Agarwal, A., Saleh, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79, 829–843. https://doi.org/10.1016/S0015-0282(02)04948-8

Aitken, R.J., Buckingham, D.W., Brindle, J., Gomez, E., Baker, H.W.G., Irvine, D.S., 1995. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. Hum. Reprod. 10, 2061–2071. https://doi.org/10.1093/oxfordjournals.humrep.a136237

Aitken, R.J., Buckingham, D.W., West, K.M., 1992. Reactive oxygen species and human spermatozoa: Analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J. Cell. Physiol. 151, 466–477. https://doi.org/10.1002/jcp.1041510305

Aitken, R.J., Fisher, H.M., Fulton, N., Gomez, E., Knox, W., Lewis, B., Irvine, S., 1997. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. Mol. Reprod. Dev. 47, 468–482. https://doi.org/10.1002/(SICI)1098-2795(199708)47:4<468::AID-MRD14>3.0.CO;2-S

Aitken, R John, Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function - In sickness and in health. J. Androl. https://doi.org/10.2164/jandrol.112.016535

Aitken, R. John, Whiting, S., De Iuliis, G.N., McClymont, S., Mitchell, L.A.,

Baker, M.A., 2012. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. J. Biol. Chem. 287, 33048–33060. https://doi.org/10.1074/jbc.M112.366690

Alvarez, J.G., Storey, B.T., 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 23, 77–90. https://doi.org/10.1002/mrd.1120230108

ALVAREZ, J.G., TOUCHSTONE, J.C., BLASCO, L., STOREY, B.T., 1987. Spontaneous Lipid Peroxidation and Production of Hydrogen Peroxide and Superoxide in Human Spermatozoa Superoxide Dismutase as Major Enzyme Protectant Against Oxygen Toxicity. J. Androl. 8, 338–348. https://doi.org/10.1002/j.1939-4640.1987.tb00973.x

Amaral, A., Paiva, C., Attardo Parrinello, C., Estanyol, J.M., Ballescà, J.L., Ramalho-Santos, J., Oliva, R., 2014. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. J. Proteome Res. 13, 5670–5684. https://doi.org/10.1021/pr500652y

Amaral, A., Ramalho-Santos, J., 2010. Assessment of mitochondrial potential: Implications for the correct monitoring of human sperm function. Int. J. Androl. 33, 180–186. https://doi.org/10.1111/j.1365-2605.2009.00987.x

Araújo, A.J. de, Lima, J.S. de, Moreira, J.C., Jacob, S. do C., Soares, M. de O., Monteiro, M.C.M., Amaral, A.M. do, Kubota, A., Meyer, A., Cosenza, C.A.N., Neves, C. das, Markowitz, S., 2007. Exposição múltipla a agrotóxicos e efeitos à saúde: estudo transversal em amostra de 102 trabalhadores rurais, Nova Friburgo, RJ. Cien. Saude Colet. 12, 115–130.

https://doi.org/10.1590/S1413-81232007000100015

Bedu-Addo, K., Costello, S., Harper, C., Machado-Oliveira, G., Lefievre, L., Ford, C., Barratt, C., Publicover, S., 2008. Mobilisation of stored calcium in the neck region of human sperm - A mechanism for regulation of flagellar activity. Int. J. Dev. Biol. 52, 615–626. https://doi.org/10.1387/ijdb.072535kb

Benton, L., Shan, L.X., Hardy, M.P., 1995. Differentiation of adult Leydig cells. J. Steroid Biochem. Mol. Biol. 53, 61–68. https://doi.org/10.1016/0960-0760(95)00022-R

Bertero, E., Maack, C., 2018. Calcium signaling and reactive oxygen species in Mitochondria. Circ. Res. 122, 1460–1478. https://doi.org/10.1161/CIRCRESAHA.118.310082

Blair, A., Sandler, D., Thomas, K., Hoppin, J.A., Kamel, F., Coble, J., Lee, W.J., Rusiecki, J., Knott, C., Dosemeci, M., Lynch, C.F., Lubin, J., Alavanja, M., 2005. Disease and injury among participants in the Agricultural Health Study. J. Agric. Saf. Health.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3

Breininger, E., Dubois, D., Pereyra, V.E., Rodriguez, P.C., Satorre, M.M., Cetica, P.D., 2017. Participation of phosphofructokinase, malate dehydrogenase and isocitrate dehydrogenase in capacitation and acrosome reaction of boar spermatozoa. Reprod. Domest. Anim. 52, 731–740. https://doi.org/10.1111/rda.12973

Buege, J.A., Aust, S.D., 1975. Microsomal lipid peroxidation. Methods Enzymol.

71, 012004. https://doi.org/10.1088/1742-6596/71/1/012004

Chakraborty, J., Nelson, L., 1976. Comparative study of cholinesterase distribution in the spermatozoa of some mammalian species. Biol. Reprod. 15, 579–585. https://doi.org/10.1093/biolreprod/15.5.579

Chakraborty, J., Nelson, L., 1974. Cholinesterase distribution during spermatid differentiation and spermatozoal maturation in the white mouse. Reproduction 38, 359–367. https://doi.org/10.1530/jrf.0.0380359

Chavarro, J.E., Toth, T.L., Wright, D.L., Meeker, J.D., Hauser, R., 2010. Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. Fertil. Steril. 93, 2222–2231. https://doi.org/10.1016/j.fertnstert.2009.01.100

Childress, J.J., Somero, G.N., 1979. Depth-related enzymic activities in muscle, brain and heart of deep-living pelagic marine teleosts. Mar. Biol. 52, 273–283. https://doi.org/10.1007/BF00398141

Cordova, D., Benner, E.A., Sacher, M.D., Rauh, J.J., Sopa, J.S., Lahm, G.P., Selby, T.P., Stevenson, T.M., Flexner, L., Gutteridge, S., Rhoades, D.F., Wu, L., Smith, R.M., Tao, Y., 2006. Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. Pestic. Biochem. Physiol. 84, 196–214. https://doi.org/10.1016/j.pestbp.2005.07.005

Costello, S., Michelangeli, F., Nash, K., Lefievre, L., Morris, J., Machado-Oliveira, G., Barratt, C., Kirkman-Brown, J., Publicover, S., 2009. Ca2+-stores in sperm: Their identities and functions. Reproduction 138, 425–437. https://doi.org/10.1530/REP-09-0134

De Lamirande, E., Gagnon, C., 1995. Impact of reactive oxygen species on

spermatozoa: A balancing act between beneficial and detrimental effects. Hum. Reprod. 10, 15–21. https://doi.org/10.1093/humrep/10.suppl_1.15

Du Pont, 2016. Benevia®.

Dwivedi, C., Long, N.J., 1989. Effect of cholinergic agents on human spermatozoa motility. Biochem. Med. Metab. Biol. 42, 66–70. https://doi.org/10.1016/0885-4505(89)90042-X

Erthal, R., Siervo, G., Staurengo-Ferrari, L., Fattori, V., Pescim, R., Verri, W., Fernandes, G., 2020. Impairment of postnatal epididymal development and immune microenvironment following administration of low doses of malathion during juvenile and peripubertal periods of rats. Hum. Exp. Toxicol. 39, 1487–1496. https://doi.org/10.1177/0960327120930076

Erthal, R.P., Staurengo-Ferrari, L., Fattori, V., Luiz, K.G., Cunha, F.Q., Pescim, R.R., Cecchini, R., Verri, W.A., Guarnier, F.A., Alves Fernandes, G.S., 2020. Exposure to low doses of malathion during juvenile and peripubertal periods impairs testicular and sperm parameters in rats: Role of oxidative stress and testosterone. Reprod. Toxicol. 96, 17–26. https://doi.org/10.1016/j.reprotox.2020.05.013

Gavella, M., Lipovac, V., 1992. Nadh-dependent oxidoreductase (diaphorase) activity and isozyme pattern of sperm in infertile men. Syst. Biol. Reprod. Med. 28, 135–141. https://doi.org/10.3109/01485019208987691

Glock, G.E., McLean, P., 1953. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55, 400–408. https://doi.org/10.1042/bj0550400

Golub, M.S., Collman, G.W., Foster, P.M.D., Kimmel, C.A., Rajpert-De-Meyts,

E., Reiter, E.O., Sharpe, R.M., Skakkebaek, N.E., Toppari, J., 2008. Public health implications of altered puberty timing. Pediatrics 121. https://doi.org/10.1542/peds.2007-1813G

Harper, C. V., Barratt, C.L.R., Publicover, S.J., 2004. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca2+]i oscillations and cyclical transitions in flagellar beating. J. Biol. Chem. 279, 46315–46325. https://doi.org/10.1074/jbc.M401194200

Ho, H.C., Suarez, S.S., 2003. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. Biol. Reprod. 68, 1590–1596. https://doi.org/10.1095/biolreprod.102.011320

Jabłońska-Trypuć, A., Wołejko, E., Wydro, U., Butarewicz, A., 2017. The impact of pesticides on oxidative stress level in human organism and their activity as an endocrine disruptor. J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes 52, 483–494. https://doi.org/10.1080/03601234.2017.1303322

Jeulin, C., Soufir, J.C., Weber, P., Laval-Martin, D., Calvayrac, R., 1989. Catalase activity in human spermatozoa and seminal plasma. Gamete Res. 24, 185–196. https://doi.org/10.1002/mrd.1120240206

Jimenez-Gonzalez, C., Michelangeli, F., Harper, C. V., Barratt, C.L.R., Publicover, S.J., 2006. Calcium signalling in human spermatozoa: A specialized “toolkit” of channels, transporters and stores. Hum. Reprod. Update 12, 253–267. https://doi.org/10.1093/humupd/dmi050

Kao, S.H., Chao, H.T., Chen, H.W., Hwang, T.I.S., Liao, T.L., Wei, Y.H., 2008.

Increase of oxidative stress in human sperm with lower motility. Fertil. Steril. 89, 1183–1190. https://doi.org/10.1016/j.fertnstert.2007.05.029

Ko, E.Y., Sabanegh, E.S., Agarwal, A., 2014. Male infertility testing: Reactive oxygen species and antioxidant capacity. Fertil. Steril. 102, 1518–1527. https://doi.org/10.1016/j.fertnstert.2014.10.020

Koppers, A.J., Mitchell, L.A., Wang, P., Lin, M., Aitken, R.J., 2011. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. Biochem. J. 436, 687–698. https://doi.org/10.1042/BJ20110114

Lahm, G.P., Selby, T.P., Freudenberger, J.H., Stevenson, T.M., Myers, B.J., Seburyamo, G., Smith, B.K., Flexner, L., Clark, C.E., Cordova, D., 2005. Insecticidal anthranilic diamides: A new class of potent ryanodine receptor activators. Bioorganic Med. Chem. Lett. https://doi.org/10.1016/j.bmcl.2005.08.034

Li, Y., Li, Y.H., Zhou, X., Wu, B., Chen, J.P., Wang, Z.K., Wang, X., Shi, H.J., Li, R.S., 2018. DNA hydroxymethylation rate in the AChE and HoxC4 promoter associated with human sperm quality. Andrologia 50, e12963. https://doi.org/10.1111/and.12963

Lushchak, O. V., Kubrak, O.I., Lozinsky, O. V., Storey, J.M., Storey, K.B., Lushchak, V.I., 2009. Chromium(III) induces oxidative stress in goldfish liver and kidney. Aquat. Toxicol. 93, 45–52. https://doi.org/10.1016/j.aquatox.2009.03.007

Mendoza, F.J., Perez-Marin, C.C., Luis, G.M., Madueño, J.A., Henley, C., Aguilera-Tejero, E., Rodriguez, M., 2012. Localization, distribution, and

function of the calcium-sensing receptor in sperm. J. Androl. 33, 96–104. https://doi.org/10.2164/jandrol.110.011254

Minárik, P., Tomaásková, N., Kollárová, M., Antalík, M., 2002. Malate Dehydrogenases - Structure and function. Gen. Physiol. Biophys. 21, 257–265.

Mukai, C., Okuno, M., 2004. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol. Reprod. 71, 540–547. https://doi.org/10.1095/biolreprod.103.026054

Nascimento, J.M., Shi, L.Z., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E.L., Berns, M.W., 2008. Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. J. Cell. Physiol. 217, 745–751. https://doi.org/10.1002/jcp.21549

Ogo, F.M., de Lion Siervo, G.E.M., Staurengo-Ferrari, L., de Oliveira Mendes, L., Luchetta, N.R., Vieira, H.R., Fattori, V., Verri, W.A., Scarano, W.R., Fernandes, G.S.A., 2018. Bisphenol A Exposure Impairs Epididymal Development during the Peripubertal Period of Rats: Inflammatory Profile and Tissue Changes. Basic Clin. Pharmacol. Toxicol. 122, 262–270. https://doi.org/10.1111/bcpt.12894

Ogo, F.M., Siervo, G.E.M.L., Gonçalves, G.D., Cecchini, R., Guarnier, F.A., Anselmo-Franci, J.A., Fernandes, G.S.A., 2017. Low doses of bisphenol A can impair postnatal testicular development directly, without affecting hormonal or oxidative stress levels. Reprod. Fertil. Dev. 29, 2245–2254. https://doi.org/10.1071/RD16432

Ojeda, S.R., Andrews, W.W., Advis, J.P., White, S.S., 1980. Recent advances in the endocrinology of puberty. Endocr. Rev. 1, 228–257. https://doi.org/10.1210/edrv-1-3-228

Podestá, E.J., Rivarola, M.A., 1974. Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development. Endocrinology 95, 455–461. https://doi.org/10.1210/endo-95-2-455

SIES, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215, 213–219. https://doi.org/10.1111/j.1432-1033.1993.tb18025.x

Silva, E.J.R., Vendramini, V., Restelli, A., Bertolla, R.P., Kempinas, W.G., Avellar, M.C.W., 2014. Impact of adrenalectomy and dexamethasone treatment on testicular morphology and sperm parameters in rats: insights into the adrenal control of male reproduction. Andrology 2, 835–846. https://doi.org/10.1111/j.2047-2927.2014.00228.x

Slimen, S., Saloua, E.F., Najoua, G., 2014. Oxidative stress and cytotoxic potential of anticholinesterase insecticide, malathion in reproductive toxicology of male adolescent mice after acute exposure. Iran. J. Basic Med. Sci. 17, 522–530. https://doi.org/10.22038/ijbms.2014.3032

Staldoni de Oliveira, V., Gomes Castro, A.J., Marins, K., Bittencourt Mendes, A.K., Araújo Leite, G.A., Zamoner, A., Van Der Kraak, G., Mena Barreto Silva, F.R., 2020. Pyriproxyfen induces intracellular calcium overload and alters antioxidant defenses in Danio rerio testis that may influence ongoing spermatogenesis. Environ. Pollut. 116055. https://doi.org/10.1016/j.envpol.2020.116055

Tremellen, K., 2008. Oxidative stress and male infertility--a clinical perspective.

Hum. Reprod. Update. https://doi.org/10.1093/humupd/dmn004

Vermouth, N.T., Carriazo, C.S., Ponce, R.H., Blanco, A., 1986. Lactate dehydrogenase X, malate dehydrogenase and total protein in rat spermatozoa during epididymal transit. Comp. Biochem. Physiol. -- Part B Biochem. 83, 381–384. https://doi.org/10.1016/0305-0491(86)90384-6

Zhang, G., Wang, Z., Ling, X., Zou, P., Yang, H., Chen, Q., Zhou, N., Sun, L., Gao, J., Zhou, Z., Cao, J., Ao, L., 2016. Mitochondrial biomarkers reflect semen quality: Results from the MARCHS study in chongqing, China. PLoS One 11, 1–14. https://doi.org/10.1371/journal.pone.0168823

Zheng, H., Zhou, X., Li, D.K., Yang, F., Pan, H., Li, T., Miao, M., Li, R., Yuan, W., 2017. Genome-wide alteration in DNA hydroxymethylation in the sperm from bisphenol A-exposed men. PLoS One 12, 1–21. https://doi.org/10.1371/journal.pone.0178535

ARTIGO 2

EXPOSE TO LOW DOSES OF CYANTRANILIPROLE DURING PERIPUBERTAL PERIOD IMPAIRS TESTICULAR AND SPERM PARAMETERS IN PUBERTAL AND ADULT WISTAR RATS

Artigo será submetido à revista — “Toxicology”.
ISSN: 0300-483x;
F.I. 2019: 4.0
Qualis CAPES 2013 - 2016 (Medicina II): A.2

EXPOSE TO LOW DOSES OF CYANTRANILIPROLE DURING PERIPUBERTAL PERIOD IMPAIRS TESTICULAR AND SPERM PARAMETERS IN PUBERTAL AND ADULT WISTAR RATS

Suellen Ribeiro da Silva Scarton[1,2], Felipe Tsuzuki[1], Dayane Priscila dos Santos[1,2], Fernando Antônio Briere[4], Matheus Felipe Zazula[3], Andréa Name Colado Simão, Ana Tereza Bittencourt Guimarães[4], Célia Cristina Leme Beu[4], Glaura Scantamburlo Alves Fernandes[1*]

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

[2]Department of Pathological Sciences, Biological Sciences Center, State University of Londrina – UEL, Rodovia Celso Garcia Cid, PR 445, Postal code: 86057-970, Londrina, Parana, Brazil

[3]Department of Cell Biology, Biological Sciences Sector - Federal University of Paraná - UFPR, Avenida Cel. Francisco H. dos Santos, 100, Postal code: 81530-000, Curitiba, Paraná, Brazil

[4]Medical and Pharmaceutical Sciences Center, State University of Western Paraná - UNIOESTE, Universitária street, 1619, PR, Postal code: 85819-110, Cascavel, Paraná, Brazil

*Corresponding author

Email address: glaura@uel.br

Abstract

Brazil is one of the countries that most uses pesticides in the world and has legislation favorable to the release of compounds with little known action in the mammalian organism or prohibited in other regions of the world. The objective of this work was to evaluate the effect of low and high doses of the insecticide Cyantraniliprole on male reproductive parameters of Wistar rats at puberty and adulthood. DPN21 males were distributed into immediate groups (n = 12 per group): 10 mg/kg - LD, 150 mg/kg - HD of cyantraniliprole and C, only water. Treatment was started in the DPN25, via intragastric. The euthanasia of 06 animals in each group occurred by anesthetic saturation in the DPN67. The other 06 animals in each group were kept alive for another 42 days, without receiving cyantraniliprole, originating the recovery groups: LDR, HDR and CR, these animals were sacrificed by anesthetic saturation in DPN110. Plasma was collected from all groups for determination of testosterone concentration. The testis was weighed and used for sperm count, evaluation of the antioxidant system and histological and stereological parameters. Sperm from the vas deferens were used for morphological evaluation. The activity of the antioxidant system between the exposure and recovery groups was similar, however in vitro testosterone production was increased in LD and HD and decreased in LDR and HDR, the number of Sertoli cells decreased in all groups that received cyantraniliprole both in exposure and recovery phases. Phase I-V of the seminiferous epithelium cycle was increased and phase VII-VIII decreased in groups that received treatment in both periods. The proportion of lumen and stroma was greater, and the epithelium smaller, only in LD, however, the number of sperm per gram of testis and daily sperm production was lower in animals that

received both doses of cyantraniliprole, regardless of testicular phase. to experiment. As a consequence, the number of normal sperm was lower in LD, HD, LDR and HDR, the main morphological abnormalities observed were: lack of curvature and isolated heads, broken and curled tails and lack of acrosome integrity. The data reveal that the damage caused by cyantraniliprole in puberty was maintained or accentuated, even after the recovery period, that is, in adulthood.

Keywords: insecticide, reproduction, Sertoli cell, testis.

1.0. Introduction

In the last decades, studies have demonstrated the negative effect of different classes of insecticides on male fertility (Erthal et al., 2020; Mehrpour et al., 2014; Queiroz and Waissmann, 2006; Zhang et al., 2020), but with the advance of modern agriculture new substances have been released annually. Cyantraniliprole is a synthetic insecticide, derived from the *Ryania speciosa* plant, belonging to the class of anthranilic diamides (Lahm et al., 2005), which regulates the release of intracellular Ca^{2+} ($[Ca^{2+}]i$) through Ca^{2+} channels of the ryanodine type (RyR) (Cordova et al., 2006). The vast majority of insecticides with malefic potential on the male genital tract act by deregulating the action of hormones (Green et al., 2021) or decreasing the protective capacity of the tissue's antioxidant system by increasing the reactive oxygen species (ROS) (Agarwal et al., 2006; Kefer et al., 2009; Moazamian et al., 2015). ROS are highly reactive oxidizing agents and include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (ROO) and hydroxyl (OH) radicals (Aitken et al., 1992).

Oxidative stress (OS) are recognized as an important cause of male infertility by decreasing the quality of sperm parameters, attacking the fluidity of the sperm plasma membrane and the integrity of sperm DNA (Agarwal et al., 2003; Tremellen, 2008). DNA damage could accelerate the process of germ cell apoptosis, resulting in the decline in sperm counts associated with male infertility (KAO *et al.*, 2008; KO; SABANEGH; AGARWAL, 2014). The combined effects of exposure to insecticides and oxidative stress may be more severe in the infancy and juvenile periods, whereas these periods represent a windows of vulnerability for the postnatal development of the male reproductive tract (Golub et al., 2008). It is known that during the periods of childhood and youth (PND 8-35) the primary

androgens are produced (Podestá and Rivarola, 1974), and between PND 28 and 56 the Leydig cells are differentiated (Benton et al., 1995). In the peripubertal period (PND 35-65), the hypothalamic-pituitary-testis axis matures and Leydig cells start producing testosterone (Golub et al., 2008). Although numerous studies have been performed to elucidate the role of androgens in the initiation and maintenance of spermatogenesis, the molecular pathways that link androgen signaling to the control of germ cell development are still not completely understood (Kamińska et al., 2020). However the disturbance of these processes causes damage to spermatogenesis and steroidogenesis and can persist in the period of sexual maturity, that is, in adult life (Slimen et al., 2014).

There are important effects of the testosterone produced by Leydig cells on the developing testicle itself, notably to stimulate the proliferation and determination of the number of Sertoli cells (Johnston et al., 2004). Postnatally, the Sertoli cells are essential for the development and maintenance of spermatogenesis through direct interactions with the developing germ cells, and in the adult animal, the overall germ cell number is dependent upon Sertoli cell number (França et al., 2016; Orth et al., 1988). Furthermore, is activity, function and survival of adult Leydig cells is likely are dependent on the continuous presence of Sertoli cells. Therefore, the regulation of the proliferation of Sertoli cells and activity during development and in adult animal is crucial for normal adult fertility (Baker et al., 2003). Sertoli cells also play key roles in signaling in the testis by serving as the targets for FSH and testosterone and by transducing those endocrine signals and other cellular cues into paracrine regulation of germ cells (Griswold and McLean, 2006). Both the response of gene expression to FSH and testosterone and the expression of growth factors or other signaling

molecular vary with testis development and with the stage of the cycle of the seminiferous epithelium revealing the requirement for a complex and carefully controlled gene network (França et al., 2016). Many toxics acts directly on Sertoli cells and, due to the importance of these cells on the maturation of the germinal epithelium histopathological changes can be observed in the germ cells. These changes include the detachment and desquamation of seminiferous germ cells epithelium, failure or delay in germ cell maturation, incomplete sperm, and increased germ cell death (Murphy and Richburg, 2014). Since we did not find any study showing the effect of cyantraniliprole on the male reproductive tract, the objective of this work was to evaluate at pubertal and adulthood age the effects of exposure to cyantraniliprole on the testis of Wistar rats exported during the peripubertal and juvenile period.

2.0. Material and methods

2.1. Animals and experimental conditions

Male Wistar rats, DPN 21 were obtained by Central Bioterium of the State University of Western Paraná – UNIOESTE, and were acclimated to the new environment at the Laboratory of Toxicology and Metabolic Dysfunction of Reproduction of the State University of Londrina - UEL, in polypropylene boxes (43 x 30 x15) (4 animals per box) under 12 h light / dark cycle, room temperature ~ 23 °C, with free access to water and standard feed. Body weight, feed, and water consumption were measured twice a week. Animal care and handling procedures were following the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised in 1978) and with

the approval of the Ethics in Use Committee of Animals of the State University of Londrina (OF. CIRC. CEUA nº134 / 2017, process n° 21106.2017.24).

2.2. Experimental design

The animals were distributed in immediate groups (n = 12): Low dose – LD (cyantraniliprole 10 mg/kg b.w – i.g.) or High dose - HD (cyantraniliprole 150 mg/kg b.w - i.g.) diluted in water, or Control - C (only water - i.g.). The animals were submitted to the experimental design from DPN 25 to the DPN 66 and six animals per group were euthanized at DPN 67 by anesthetic saturation with Isoforine® 1.28% (Cristália, Itapira, SP, BR). According to Ojeda *et al.*, (1980) these days correspond to the peripubertal periods in male rats. The other six animals in each group were kept alive for another 42 days without receiving the corresponding doses of cyantraniliprole to verify the post-exposure effect to the toxic. The groups originated by these animals were: control recovery - CR, low dose recovery - LDR, and high dose recovery - HDR. The euthanasia was done on DPN 110, as previously described for DPN 67. Once that, nowadays there is no knowledge on the reproductive toxicity of this chemical available in the literature, the doses used in this present experiment were based on the Safety Data Sheet information. The DL_{50} reported to adult male rat, orally, is > 5000 mg/kg (Safety Data Sheet, 2016).

2.3. Preparation of Cyantraniliprole

Cyantraniliprole (3-Bromo-1- (3-chloro-2-pyridinyl) -N- [4-cyano-2-methyl-6 - [(methylamino) carbonyl] phenyl] -1 H-pyrazole-5-carboxamide; 10 , 0% w / v) (10% pure, Du Pont do Brasil, S.A.), was received as a donation from the Center for Agricultural Sciences of the State University of Londrina - UEL. Tap water was used as a vehicle for the dilution of the toxic agent.

2.4. Plasma blood, testis and sperm collection

Blood was collected in the presence of anticoagulant heparin (Hemofol®, São Paulo, Brazil) for the determination of plasma testosterone concentrations (n=06 rats per group). The right testis were removed, weighed and used for the evaluation of oxidative stress (n=06 per group) or in vitro testosterone production (n=06 per group) while the left testes were used for sperm counts (n=06 per group) or histopathological analysis (n=05 per group). Spermatozoa from the right and left vas deferens were used for sperm morphology (n=06 per group) and acrosome integrity analysis (n=06 per group), respectively.

2.5. Plasma testosterone, concentration of intratesticular testosterone (TI), and in vitro testosterone production (TIV)

Blood plasma was obtained via centrifugation at 3000 *g* for 15 min at 4°C and stored at −20°C until assayed via immunoassay. To measure the intratesticular testosterone concentration and testosterone production in vitro, the testicular albuginous tunic was removed and two 50 mg portions of the testicular parenchyma were placed in two separate microtubes, then 1 ml of 199 buffered medium was added (GIBCO®, Life Technologies Corporation, CA). To test testosterone production in vitro, one of the samples was stimulated with 2 ml of 100 mIU/ml HCG (Chorulon, MSD Animal Health). Both were incubated for 2h at 34 °C. After the incubation period, the material was centrifuged for 5 min at 10,000 g at 8 °C. The supernatant was used to measure the testosterone concentration. All tests were measured by chemiluminescence (2nd Generation Testosterone. Architect System, Abbott, Wiesbaden, Germany), according to the

manufacturer's recommendations. The intra-assay coefficient of variation and minimum sensitivity of the assay was 4.6 % and 0.15 nmol/L, respectively.

2.6. Histological processing

The left testes were removed and fixed in Modified Davidson's fluid (30% of a 37–40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H_2O) for 6 h and post-fixed with PFA 4% for 18 h at 4 °C as described by WANG *et al.*, (2016). The testes were embedded in Paraffin wax® (SIGMA Life Science). Tree nonconsecutive sections (7m thick) per animal, separate by 30 µm distance, were obtained, mounted on glass slides and stained with hematoxylin and eosin (HE).

2.6.1. Histopathological analysis in testis

For the stereological analysis of the testis, sections were photocumented with the aid of an Olympus DP71 microscope coupled to the Olympus BX60® camera (Olympus Corporation, Tokyo, Japan) in 20x magnification. Then, 10 photo microphotographs of each animal were superimposed with the Weibel grid, and 168 points were counted to differentiate the percentage of stromal, germinal epithelium and luminal compartments of the seminiferous tubules. Histopathological analysis was performed from the same images to identify the presence of abnormal seminiferous tubules (vacuolization of the seminiferous epithelium, immature cells in the light of the seminiferous tubule, absence of germ cells in the seminiferous tubule) (Favareto et al., 2011).

2.6.2. Number of Leydig cells

According to Guerra *et al.*, (2017) the number of Leydig cell nuclei was counted in 10 random fields of interstitial tissue in each testis section per rat under a light microscope at 400x magnification. The Leydig cell morphology was identified as described by Teerds and Huhtaniemi (Teerds and Huhtaniemi, 2015).

2.6.3. Number of Sertoli cells

The number of Sertoli cell nuclei was determined in 20 cross-sections of the seminiferous tubules per testis in each rat, under a light microscope at 400x magnification (Nassr et al., 2010).

2.6.4. Spermatogenesis kinetics

One hundred random tubular sections per animal were classified into four categories, namely in stages I-VI, VII-VIII, IX-XIII, and XIV, of the seminiferous epithelium cycle, according to Leblond and Clermont (1952), under a light microscope (Olympus Bx60®) at 100x and 400x magnification. This analysis allows the assessment of the proportion of staging of the seminiferous tubules.

2.6.5. Daily sperm production

To evaluate daily sperm production (DSP), the left testes were decapsulated, weighed, and homogenized, as described previously (Robb et al., 1978), with some modifications as described by Siervo et al. (2015). After dilution of the homogenate, a small sample volume was transferred to a Neubauer chamber (four fields per animal) for the counting of homogenization-resistant

spermatids (stage 19 of spermatogenesis). To calculate DSP, the concentration of spermatids per testis was divided by 6.1, which refers to the number of days for which the mature spermatids are present in the seminiferous epithelium.

2.6.5. Sperm morphology

The contents of the vas deferens were removed via internal rinsing with 1.0 mL of 10 % formol saline. Histological slides were prepared from this solution and observed using an Olympus Bx60® microscope (400x magnification). Two hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached), and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached). This analysis was performed as described by Erthal *et al.*, (2020).

2.6.8. Acrosome integrity

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

3. Biomarkers of oxidative stress

The right testes were homogenized in 2 mL of phosphate buffer (pH 7.4) and centrifuged at 9,500 g for 10 min at 4ºC. The protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as a standard (Bradford, 1976). The supernatant was separated and used for the following analyzes.

3.1. Concentration of GSH and other non-protein thiols (NP-SH)

The concentration of GSH and other non-protein thiols (NP-SH) in the testes were determined by the interaction of non-protein thiols with DTNB and the formation of a yellowish chromophore. To carry out the test, it was necessary to precipitate the proteins in the sample by adding 30% TCA (1: 5 v / v) and centrifuging at 7000 RCF (g) at 4 ºC for 10 minutes. The supernatant was used for the quantification of thiols with the addition of 250 µM of DTNB and the absorbance by the generation of the chromophore was measured at 415 nm. Values were expressed in nM thiols.mg protein^{-1} (Sedlak and Lindsay, 1968).

3.1.2 Lipid peroxidation index - LPO

The lipid peroxidation index (LPO) of the testes and adipose tissue was determined by the generation of complexes between Fe^{+2} and xylenol orange and the formation of a chromophore stabilized by butylated hydroxytoluene. The absorbance by the generation of the chromophore was measured at 560 nm. Values were expressed in nM hydroperoxides.mg protein^{-1} (Jiang et al., 1991).

3.1.3. Glutathione S-transferase activity - GST

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

3.1.4. Glutathione peroxidase activity - GPx

The enzymatic activity of glutathione peroxidase (GPx - EC 1.11.1.9) of the testes was determined through the formation of GSSG from the reduction of GSH in the consumption of H_2O_2. The reduction of the absorbance by oxidation of NADPH was monitored at 340 nm (RS: 100 mM potassium phosphate buffer pH 7.0; 2 mM sodium azide; 200 μM NADPH; 2 mM GSH; 1 U / mL GR; 500 μM H_2O_2). Values were expressed in μM NADPH oxidized.min^{-1} x mg $protein^{-1}$ (Wendell, 1981).

3.2. Statistical analysis

All variables were analyzed by the normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) and those that were in agreement with such assumptions, were analyzed by One-Way Analysis of Variance (ANOVA) by period (exposition and recuperation) follow by the post-hoc Tukey-HSD test, to examine the effect of supplementation. When the assumptions were not in agreement, the Kruskal-Wallis test was performed followed by post-hoc Dunn test. All analyses were performed with a level of significance $\alpha = 0.05$.

The matrices of hormonal influence (Leydig cells, Sertoli cells, testosterone plasmatic, intratesticular and in vitro), spermatogenesis kinetic (I-VI, VII-VIII, IX-XIII, XVI cells, and lumen, stroma and epithelium proportion, and sperm by testicle weight and sperm production by day), testicle Antioxidant System (protein, LPO, NP-SH, GPx, and GST), were standardized (z-score), and from these matrices performed Permutational Multivariate Analysis of Variance (PERMANOVA) using the respective Euclidean distance matrices, established as fixed factors the treatments (Control, 10 mg and 150 mg) in immediate and recuperation periods, using “vegan” package and “adonis2” function. The pairwise comparisons for all pairs of levels of the fixed factor were performed by using Permutational MANOVA with Bonferroni correction method (“EcolUtils” package, adonis.pair function) . The results graphical representations were performed by resulting biplot of Principal Coordinate Analysis (“ape” package, pcoa function). All analyses were performed in software R (R Core Team, 2020).

4.0. Results

4.1. Plasma testosterone, concentration of intratesticular testosterone (TI), and in vitro testosterone production (TIV)

The plasma testosterone concentration was not different between the immediate groups (table 1) or between the groups that underwent recovery (table 2). The concentration of intratesticular testosterone in the exposed animals was not different between groups (table 1). However, animals in the LDR group had a lower intratesticular testosterone concentration compared to the control group. The HDR group was statistically equal to the LDR and the C group (table 2). In vitro testosterone production was higher in the HD group compared to the

exposed C, while the LD group was statistically equal to HD and the C group (table 1). The LDR had lower testosterone production in vitro when compared to the CR group. However, in the HDR this parameter had similar production to LDR and the CR (table 2).

4.1.2. Histopathological analysis in testis, number of Leydig and Sertoli cells

The stereology of the testis in the immediate groups revealed a significant increase in the lumen compartment in the LD when compared to the C and HD groups. The stromal compartment was increased by LD and HD compared to the C. The seminiferous epithelium compartment was LD smaller when compared to HD and compared to the C group. The HD seminiferous epithelium compartment was statistically smaller than in the control (table 1.) No statistical differences were observed when comparing the lumen, stroma and epithelium compartments of the groups that underwent recovery (table 2). Histopathological analysis of the testicles showed that the animals that received cyantraniliprole, both during the immediate period, and those that underwent recovery (Fig. 4), presented abnormalities in the seminiferous tubules such as epithelium vacuolization (Fig.4C, E), immature cells in the lumen of the seminiferous tubule (Fig. 4F) and rupture of the tissue with loss of germ cells (Fig. 4B, C,E).

The number of Leydig cells was not different between the immediate groups (table 1) or among the groups that went through the recovery period (table 2). The number of Sertoli cells was lower in the LD and HD groups compared to the control. However, LD and HD had a similar number of Sertoli cells (table 1). When verifying the number of Sertoli cells in the recovery groups, it was observed

that the LDR group had a lower number of cells when compared to the HDR and CR groups. The HDR group also had a lower number of Sertoli cells when compared to the CR (table 2).

4.1.3 Spermatogenesis kinetic, daily sperm production, sperm morphology and acrosome integrity

The evaluation of spermatogenic kinetics for the immediate and recovery groups showed that the number of seminiferous tubules in stage I - VI has significantly increased by LD, HD and LDR, HDR when compared to the C and CR, while stage VII - VIII has statistically decreased by LD, HD and LDR, HDR when compared to the C and CR groups. Stages IX - XIII and XIV were statistically equal among the groups of immediate and recovery (table 1 and 2).

In relation to daily production of sperm and the number of sperm per gram of testis, there was a significantly decreased in animals LD, LDR and HD, HDR in relation to the C and CR groups (table 1 and 2).

The results of the morphological evaluation and the integrity of the sperm acrosome are shown in table 3. The animals of the immediate and recovery period, both the dose of LD, HD or LDR and HDR had a lower number of normal sperm compared to the C and CR group. The number of normal sperm was statistically higher in the HD group than in LD. The lack of curvature of the sperm head was greater in the LD and HD group compared to the C, but HD was greater than to LD group. After recovery, this defect was greater in HDR when compared to LDR and CR. The number of isolated sperm heads was statistically higher in the LD and HD groups compared to the C, while in HDR this parameter was higher compared to LDR and CR. Isolated tails occurred in greater numbers in

sperm from the LD and HD groups of cyantraniliprole in relation to the control, but the comparison between LD and HD revealed a statistically equal number in this parameter. LDR group had the highest number of isolated tails compared to the HDR and CR groups, however, in HDR this defect was greater than in CR. The number of broken tails was higher in the LD group compared to the control group, but it was statistically equal to the HD groups. The HD group showed a similar result to the control group. In the recovery period, LDR and HDR had more sperm with broken tail than CR. The amount of tail curled in the sperm of the LD and HD was higher when compared to the control, however, the comparison between LD and HD was statistically equal. HDR had a higher number of curled tails compared to LDR and CR. A smaller number of intact acrosomes and a greater number of acrosomes without integrity were observed in the sperm of animals LD, HD and LDR, HDR, when compared to the CR and C groups, respectively.

4.1.4. Biomarkers of oxidative stress

The concentration of GSH and other non-protein thiols (NP-SH) in the testes, as well as the activity of GPx, GST, and the occurrence of OLP was not different between the exposed group (table 1) and the recovery group (table 2).

4.2 Matrices for influence hormonal, spermatogenesis kinect and testicle antioxidant system

Were constructed a principal coordinates ordination which illustrate the dissimilarity among different variable matrices and treatments in each period (exposition and recuperation). Multivariate analysis demonstrated that overall

matrices, that represent the physiology and morphology relation on the individuals were significantly different among the treatment. As for hormonal influence matrix, it was found that the variables are not influenced by the treatments in exposition period (Fig. 2A), but we found effects on recuperation period (Fig. 2B), both treatments trend to statistical differences between the control group. Spermatogenesis kinect matrices (germ cell maturation) show statistical differences in exposition period (Fig. 3A), 10 mg and 150 mg groups being statistically different from Control group, as well as in the recuperation period (Fig. 3B). The variables related to Testicle Antioxidant System, in exposition period, are not influenced by the treatments (Fig. 1A), despite the significant effect of the 150 mg treatment compared to the control group. In recuperation period, the set of variables are not influenced by the treatments (Fig. 1B).

5.0 Discussion

The present study is the first to demonstrated that cyantraniliprole harms the maturation of germ cells of animals exposed to this compound during critical periods of development and that these damages generated in the peripubertal period remained in adulthood. This condition can be explained by the influence exerted by Sertoli cells in the testicular environment, but not by oxidative stress biomarkers. Postnatally these cells are essential for the development and maintenance of spermatogenesis through direct interactions with developing germ cells, and in the adult animal, the total number of germ cells is dependent on the number of Sertoli cells (Orth et al., 1988). In this study, the reduction of Sertoli cells in the immediate exposed animals which remained in a smaller

number in the adulthood animals, being the 10mg dose more impairment that 150mg, evidences the persistence this harm effects and the vulnerability of pubertal period.

Alteration in sperm parameters could be attributed to a direct effect on testicular tissue which leads to reproductive dysfunction, such as changes in Sertoli cells (Griswold and McLean, 2006). The sperm morphology analysis in this study showed the significantly increase of the number of abnormal sperm demonstrates a direct effect of cyantraniliprole on the spermiogenesis process, or may, be related to the reduction in the number of Sertoli cells (Siervo et al., 2015) associated the increase of abnormal head sperm to a possible effect on spermiogenesis.

In pubertal and adult age, the evaluation of spermatogenic kinetics revealed that cyantraniliprole exposure caused a blockage in the spermatogenetic process once there was an increase in the number of tubules seminiferous in stage I-VI and consequently a decrease in stages VII-VIII. In associated with results, cyantraniliprole exposure also caused a reduction in daily sperm production in both ages since it is known that in stage VII-VIII occurs the spermiation process. These are important results that shows that the administration of cyantraniliprole, in both doses, during pubertal period cause immediate and persistent damage to the spermatogenesis cycle and sperm production, respectively. Therefore, is evident the great susceptibility of the peripubertal period to toxic compounds such as the present insecticide.

On the other hand, although are possible that alterations in spermatogenesis kinetics alter the proportion of testicular compartments, this relationship occurred partially in the present work. Based on the analysis in both

age and doses, the increase in the luminal and stromal compartment with a consequent decrease in the germinal epithelium in pubertal age did not remain in adult life. Thus, it is possible that occurred a tissue readjustment during the recovery period.

Puberty generally involves the cessation of mitosis of Sertoli cells, the formation of tight junctions between adjacent Sertoli cells, and the progression of germ cells through meiosis and differentiation into spermatozoa (Griswold, 1998). Each Sertoli cell simultaneously interacts with undifferentiated spermatogonia, differentiating spermatogonia, meiotic spermatocytes, and post-meiotic spermatids in the mammalian testis, which requires extensive apicobasal polarity. Sertoli cell polarity is evident by the specific localization of tight-junction components in the blood-testis barrier (BTB) (Gao and Cheng, 2016). This arrangement favors the development of sperm morphology, as it ensures proper sperm fixation and alignment before sperm is released (Griswold and McLean, 2006). The histopathological abnormalities of the testis observed in this work lead us to believe that the junctions that form the blood-testis barrier were impaired by exposure to cyantraniliprole causing the morphological changes observed in sperm. However, the BTB is not a static ultrastructure. Instead, it undergoes extensive restructuring during the seminiferous epithelial cycle of spermatogenesis at stage VIII to allow the transit of preleptotene spermatocytes at the BTB (Mruk and Cheng, 2015). As we observed the decrease in the number of Sertoli cells and the stages of cycle VIII, in both periods, this would be a plausible explanation for the morphological abnormalities of the sperm. The transport of germ cells across the seminiferous epithelium is crucial to spermatogenesis. Its disruption causes infertility (Xiao et al., 2014).

According to Meistrich & Hess (2013), during the initial stages of morphogenic transformation, the elongated spermatids are fixed in the most apical crypts of the Sertoli cell and extend perpendicularly to the basement membrane. As the sperm cells elongate, the germ cells are transported to the deep recesses of the Sertoli cell, with their heads almost touching the nucleus of the Sertoli cell (Hess, 1990). As the last step, the Sertoli cell transports sperm from the final stage towards the lumen, where fully developed sperm are released during sperm. This dynamic mobilization of elongated spermatids is regulated by the Sertoli cell through the use of parallel microtubule tracts and motor proteins attached to the endoplasmic reticulum component of ectoplasmic specialization (Amlani and Vogl, 1988; VOGL et al., 1991). We believe that cyantraniprole has impaired the morphogenic transformation of germ cells into sperm and as a result we find acrosomes without integrity, sperm heads separated from the tails, broken and curled tails, which can lead to low fertility. Beurois *et al.*, (2020) report that spermatozoa are polarized cells with a head and a flagellum joined by the connecting piece. Head integrity is critical for normal sperm function, and head defects consistently lead to male infertility. Abnormalities of the sperm head are among the most severe and characteristic sperm defects. In addition, when creating the matrices to observe the influence of the variables, we demonstrated that the negative effects of cyantraniliprole occurred mainly on the germinal epithelium, correlating with changes in the phases of the seminiferous epithelium cycle and a decrease in the proportion of the germinal epithelium which culminated in a reduction in the daily production of sperm and morphological damage to these cells.

Sertoli cells express receptors for follicle-stimulating hormone (FSH) and testosterone (T), which are the main hormonal regulators of spermatogenesis (Sofikitis et al., 2008). The plasma and intratesticular testosterone concentration are correlated with the number of Leydig cells and they undergo expansion from PND 2 to 56. Between PND 7-14 adults, Leydig stem cells proliferate and differentiate into progenitor Leydig cells, which predominate in the testis of PND 14-21. Progenitor Leydig cells differentiate into immature Leydig cells around PND 35. Immature Leydig cells proliferate and differentiate into adult Leydig cells, starting with PND 55-90 (Chen et al., 2009; Hardy et al., 1989; Martin, 2016). In the current study, despite the higher dose of cyantraniliprole caused higher testosterone production in vitro in the immediate evaluation, the unchanged plasma testosterone, intratesticular testosterone, and in the number of Leydig cells after exposure cyantraniliprole showed that toxic was not able to interfere in the differentiation of progenitors Leydig cells in Leydig immature cells and later in adult Leydig cells.

Although cyantraniliprole did not change the antioxidant system, we observed a decrease in GST and other non-thiol proteins in the immediate period. Thiols are the most important endogenous systemic and intracellular antioxidants. Reactions of thioic compounds with different oxidants formed in vivo generate antioxidant actions. The oxidation of thiol groups on protein residues is crucial in redox signaling, a process by which a biological system responds to changes in ROS levels (DENEKE, 2001). We believe that this was the mechanism which made the animals that went through the recovery period not to present such alterations, indicating that the antioxidant system may have been modulated as a function of time without exposure to cyantraniliprole.

Conclusion

In conclusion, cyantraniliprole exposure during a critical period of development, the peripubertal period, was toxic to the male reproductive system once there was impairment of the testicular morphology and function in puberty and adulthood of rats.

Acknowledgment

The authors are grateful to CAPES (Coordinating Body for the Improvement of Postgraduate Studies in Higher Education) for providing a PhD scholarship to Scarton, S.R.S and partially financial support (Finance Code 001). This paper represents part of the PhD thesis by Scarton, S.R.S. (State University of Londrina - Brazil) under the supervision of GSA Fernandes.

Conflicts of interest

The authors declare no conflicts of interest.

References

Agarwal, A., Saleh, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79, 829–843. https://doi.org/10.1016/S0015-0282(02)04948-8

Agarwal, A., Sharma, R.K., Nallella, K.P., Thomas, A.J., Alvarez, J.G., Sikka, S.C., 2006. Reactive oxygen species as an independent marker of male factor infertility. Fertil. Steril. 86, 878–885. https://doi.org/10.1016/j.fertnstert.2006.02.111

Aitken, R.J., Buckingham, D.W., West, K.M., 1992. Reactive oxygen species and human spermatozoa: Analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J. Cell. Physiol. 151, 466–477. https://doi.org/10.1002/jcp.1041510305

Amlani, S., Vogl, A.W., 1988. Changes in the distribution of microtubules and intermediate filaments in mammalian Sertoli cells during spermatogenesis. Anat. Rec. 220, 143–160. https://doi.org/10.1002/ar.1092200206

Baker, P.J., Pakarinen, P., Huhtaniemi, I.T., Abel, M.H., Charlton, H.M., Kumar, T.R., O'Shaughnessy, P.J., 2003. Failure of normal Leydig cell development in follicle-stimulating hormone (FSH) receptor-deficient mice, but not FSHβ-deficient mice: Role for constitutive FSH receptor activity. Endocrinology 144, 138–145. https://doi.org/10.1210/en.2002-220637

Benton, L., Shan, L.X., Hardy, M.P., 1995. Differentiation of adult Leydig cells. J. Steroid Biochem. Mol. Biol. 53, 61–68. https://doi.org/10.1016/0960-0760(95)00022-R

Beurois, J., Cazin, C., Kherraf, Z., Martinez, G., Celse, T., Touré, A., Arnoult, C., Ray, P.F., Coutton, C., 2020. Genetics of teratozoospermia: Back to the

head. Best Pract. Res. Clin. Endocrinol. Metab. 34, 101473. https://doi.org/10.1016/j.beem.2020.101473

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3

Chen, H., Ge, R.S., Zirkin, B.R., 2009. Leydig cells: From stem cells to aging. Mol. Cell. Endocrinol. 306, 9–16. https://doi.org/10.1016/j.mce.2009.01.023

Cordova, D., Benner, E.A., Sacher, M.D., Rauh, J.J., Sopa, J.S., Lahm, G.P., Selby, T.P., Stevenson, T.M., Flexner, L., Gutteridge, S., Rhoades, D.F., Wu, L., Smith, R.M., Tao, Y., 2006. Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. Pestic. Biochem. Physiol. 84, 196–214. https://doi.org/10.1016/j.pestbp.2005.07.005

Erthal, R.P., Staurengo-Ferrari, L., Fattori, V., Luiz, K.G., Cunha, F.Q., Pescim, R.R., Cecchini, R., Verri, W.A., Guarnier, F.A., Alves Fernandes, G.S., 2020. Exposure to low doses of malathion during juvenile and peripubertal periods impairs testicular and sperm parameters in rats: Role of oxidative stress and testosterone. Reprod. Toxicol. 96, 17–26. https://doi.org/10.1016/j.reprotox.2020.05.013

Favareto, A.P.A., de Toledo, F.C., Kempinas, W.D.G., 2011. Paternal treatment with cisplatin impairs reproduction of adult male offspring in rats. Reprod. Toxicol. https://doi.org/10.1016/j.reprotox.2011.10.003

França, L.R., Hess, R.A., Dufour, J.M., Hofmann, M.C., Griswold, M.D., 2016. The Sertoli cell: one hundred fifty years of beauty and plasticity. Andrology 4, 189–212. https://doi.org/10.1111/andr.12165

Gao, Y., Cheng, C.Y., 2016. Does cell polarity matter during spermatogenesis? Spermatogenesis 6, e1218408. https://doi.org/10.1080/21565562.2016.1218408

Golub, M.S., Collman, G.W., Foster, P.M.D., Kimmel, C.A., Rajpert-De-Meyts, E., Reiter, E.O., Sharpe, R.M., Skakkebaek, N.E., Toppari, J., 2008. Public health implications of altered puberty timing. Pediatrics 121. https://doi.org/10.1542/peds.2007-1813G

Green, M.P., Harvey, A.J., Finger, B.J., Tarulli, G.A., 2021. Endocrine disrupting chemicals : Impacts on human fertility and fecundity during the peri-conception period. Environ. Res. 194, 110694. https://doi.org/10.1016/j.envres.2020.110694

Griswold, M.D., 1998. The central role of Sertoli cells in spermatogenesis. Semin. Cell Dev. Biol. https://doi.org/10.1006/scdb.1998.0203

Griswold, M.D., McLean, D., 2006. The sertoli cell. Knobil Neill's Physiol. Reprod. https://doi.org/10.1016/B978-012515400-0/50024-5

Guerra, M.T., Sanabria, M., Leite, G.A.A., Borges, C.S., Cucielo, M.S., Anselmo-Franci, J.A., Foster, W.G., Kempinas, W.G., 2017. Maternal exposure to butyl paraben impairs testicular structure and sperm quality on male rats. Environ. Toxicol. 32, 1273–1289. https://doi.org/10.1002/tox.22323

Hardy, M.P., Zirkin, B.R., Ewing, L.L., 1989. Kinetic studies on the development of the adult population of leydig cells in testes of the pubertal rat. Endocrinology 124, 762–770. https://doi.org/10.1210/endo-124-2-762

Hess, R. a, 1990. Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic

observations of perfusion-fixed and plastic-embedded testes. Biol. Reprod. https://doi.org/10.1095/biolreprod43.3.525

Jiang, Z.-Y., Woollard, A.C.S., Wolff, S.P., 1991. Lipid hydroperoxides measurement by oxidation of Fe2+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids 26, 853–856.

Johnston, H., Baker, P.J., Abel, M., Charlton, H.M., Jackson, G., Fleming, L., Kumar, T.R., O'Shaughnessy, P.J., 2004. Regulation of Sertoli Cell Number and Activity by Follicle-Stimulating Hormone and Androgen during Postnatal Development in the Mouse. Endocrinology 145, 318–329. https://doi.org/10.1210/en.2003-1055

Kamińska, A., Marek, S., Pardyak, L., Brzoskwinia, M., Pawlicki, P., Bilińska, B., Hejmej, A., 2020. Disruption of androgen signaling during puberty affects Notch pathway in rat seminiferous epithelium. Reprod. Biol. Endocrinol. 18, 1–14. https://doi.org/10.1186/s12958-020-00582-3

Kao, S.H., Chao, H.T., Chen, H.W., Hwang, T.I.S., Liao, T.L., Wei, Y.H., 2008. Increase of oxidative stress in human sperm with lower motility. Fertil. Steril. 89, 1183–1190. https://doi.org/10.1016/j.fertnstert.2007.05.029

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the gluthathione S-transferases. J Biol Chem 251, 6138–6188.

Kefer, J.C., Agarwal, A., Sabanegh, E., 2009. Role of antioxidants in the treatment of male infertility. Int. J. Urol. 16, 449–457. https://doi.org/10.1111/j.1442-2042.2009.02280.x

Ko, E.Y., Sabanegh, E.S., Agarwal, A., 2014. Male infertility testing: Reactive oxygen species and antioxidant capacity. Fertil. Steril. 102, 1518–1527.

https://doi.org/10.1016/j.fertnstert.2014.10.020

Lahm, G.P., Selby, T.P., Freudenberger, J.H., Stevenson, T.M., Myers, B.J., Seburyamo, G., Smith, B.K., Flexner, L., Clark, C.E., Cordova, D., 2005. Insecticidal anthranilic diamides: A new class of potent ryanodine receptor activators. Bioorganic Med. Chem. Lett. https://doi.org/10.1016/j.bmcl.2005.08.034

Martin, L.J., 2016. Cell interactions and genetic regulation that contribute to testicular Leydig cell development and differentiation. Mol. Reprod. Dev. 83, 470–487. https://doi.org/10.1002/mrd.22648

Mehrpour, O., Karrari, P., Zamani, N., Tsatsakis, A.M., Abdollahi, M., 2014. Occupational exposure to pesticides and consequences on male semen and fertility: A review. Toxicol. Lett. 230, 146–156. https://doi.org/10.1016/j.toxlet.2014.01.029

Meistrich, M.L., Hess, R.A., 2013. Assessment of spermatogenesis through staging of seminiferous tubules. Methods Mol. Biol. 927, 299–307. https://doi.org/10.1007/978-1-62703-38-0_27

Moazamian, R., Polhemus, A., Connaughton, H., Fraser, B., Whiting, S., Gharagozloo, P., Aitken, R.J., 2015. Oxidative stress and human spermatozoa: Diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. Mol. Hum. Reprod. 21, 502–515. https://doi.org/10.1093/molehr/gav014

Mruk, D.D., Cheng, C.Y., 2015. The mammalian blood-testis barrier: Its biology and regulation. Endocr. Rev. https://doi.org/10.1210/er.2014-1101

Murphy, C.J., Richburg, J.H., 2014. Implications of Sertoli cell induced germ cell apoptosis to testicular pathology. Spermatogenesis 4, e979110.

https://doi.org/10.4161/21565562.2014.979110

Nassr, A.C.C., Arena, A.C., Toledo, F.C., Bissacot, D.Z., Fernandez, C.D.B., Spinardi-Barbisan, A.L.T., Pires, P.W., Kempinas, W.G., 2010. Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. J. Toxicol. Environ. Heal. - Part A Curr. Issues 73, 952–964. https://doi.org/10.1080/15287391003751745

Ojeda, S.R., Andrews, W.W., Advis, J.P., White, S.S., 1980. Recent advances in the endocrinology of puberty. Endocr. Rev. 1, 228–257. https://doi.org/10.1210/edrv-1-3-228

Orth, J.M., Gunsalus, G.L., Lamperti, A.A., 1988. Evidence From Sertoli Cell-Depleted Rats Indicates That Sertoli Cells Produced During Perinatal Development. Endocrinology 122, 787–794.

Podestá, E.J., Rivarola, M.A., 1974. Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development. Endocrinology 95, 455–461. https://doi.org/10.1210/endo-95-2-455

Queiroz, E.K.R. de, Waissmann, W., 2006. Occupational exposure and effects on the male reproductive system. Cad. Saude Publica. https://doi.org/10.1590/S0102-311X2006000300003

Robb, G.W., Amann, R.P., Killian, G.J., 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54, 103–107. https://doi.org/10.1530/jrf.0.0540103

Sedlak, J., Lindsay, R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent. Anal. Biochem. 25, 192–205. https://doi.org/10.1016/0003-2697(68)90092-4

Siervo, G.E.M.L., Vieira, H.R., Ogo, F.M., Fernandez, C.D.B., Gonçalves, G.D., Mesquita, S.F.P., Anselmo-Franci, J.A., Cecchini, R., Guarnier, F.A., Fernandes, G.S.A., 2015. Spermatic and testicular damages in rats exposed to ethanol: Influence of lipid peroxidation but not testosterone. Toxicology. https://doi.org/10.1016/j.tox.2015.01.016

Silva, E.J.R., Vendramini, V., Restelli, A., Bertolla, R.P., Kempinas, W.G., Avellar, M.C.W., 2014. Impact of adrenalectomy and dexamethasone treatment on testicular morphology and sperm parameters in rats: insights into the adrenal control of male reproduction. Andrology 2, 835–846. https://doi.org/10.1111/j.2047-2927.2014.00228.x

Slimen, S., Saloua, E.F., Najoua, G., 2014. Oxidative stress and cytotoxic potential of anticholinesterase insecticide, malathion in reproductive toxicology of male adolescent mice after acute exposure. Iran. J. Basic Med. Sci. 17, 522–530. https://doi.org/10.22038/ijbms.2014.3032

Sofikitis, N., Giotitsas, N., Tsounapi, P., Baltogiannis, D., Giannakis, D., Pardalidis, N., 2008. Hormonal regulation of spermatogenesis and spermiogenesis. J. Steroid Biochem. Mol. Biol. https://doi.org/10.1016/j.jsbmb.2008.03.004

Teerds, K.J., Huhtaniemi, I.T., 2015. Morphological and functional maturation of Leydig cells: From rodent models to primates. Hum. Reprod. Update 21, 310–328. https://doi.org/10.1093/humupd/dmv008

Tremellen, K., 2008. Oxidative stress and male infertility--a clinical perspective. Hum. Reprod. Update. https://doi.org/10.1093/humupd/dmn004

VOGL, A.W., PFEIFFER, D.C., REDENBACH, D.M., 1991. Ectoplasmic (“Junctional”) Specializations in Mammalian Sertoli Cells: Influence on

Spermatogenic Cells. Ann. N. Y. Acad. Sci. 637, 175–202. https://doi.org/10.1111/j.1749-6632.1991.tb27310.x

Wang, H., Yang, L.L., Ji, Y.L., Chen, Y.H., Hu, J., Zhang, C., Zhang, J., Xu, D.X., 2016. Different fixative methods influence histological morphology and TUNEL staining in mouse testes. Reprod. Toxicol. 60, 53–61. https://doi.org/10.1016/j.reprotox.2016.01.006

Wendell, A., 1981. Glutathione Peroxidase, in: Methods in Enzimology. Elsevier, pp. 325–333.

Xiao, X., Mruk, D.D., Wong, C.K.C., Yan Cheng, C., 2014. Germ cell transport across the seminiferous epithelium during spermatogenesis. Physiology 29, 286–298. https://doi.org/10.1152/physiol.00001.2014

Zhang, X., Cui, W., Wang, K., Chen, R., Chen, M., Lan, K., Wei, Y., Pan, C., Lan, X., 2020. Chlorpyrifos inhibits sperm maturation and induces a decrease in mouse male fertility. Environ. Res. 188, 109785. https://doi.org/10.1016/j.envres.2020.109785

Legend of tables

Table 1

Matrices for the immediate period

Data are presented as the median, 1° and 3° quartilhes, One-way ANOVA test with a post test of Tukey or median, 1 and 3 ° quartilhes, *Kruskal-Wallis test with post hoc Dunn's test. $p < 0.05$.

Table 2

Matrices for recovery period

Data are presented as the median, 1° and 3° quartilhes, One-way ANOVA test with a post test of Tukey. $p < 0.05$.

Table 3
Effects of different doses of cyantraniliprole on immediate and recovery periods on sperm acrosome morphology and integrity

Data are presented as the median, 1° e 3° quartilhes. One-way ANOVA test with a post test of Tukey or *Kruskal-Wallis test with post hoc Dunn's test. $p < 0.05$. LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

Legend of figure

Figure 5 - Principal Coordinates Coordinates Analysis (PCoA) plot of testicle Antioxidant System matrices: A: Exposition period. B: Recuperation period.

Figure 2 - Principal Coordinates Coordinates Analysis (PCoA) plot of hormonal influence matrices: A: Exposition period. B: Recuperation period.

Figure 3 - Principal Coordinates Coordinates Analysis (PCoA) plot of Seminiferous epithelium cycle matrices: A: Exposition period. B: Recuperation period.

Figure 4 - Histopathological analysis of the testis.

In A and D, normal characteristics of the semiferiferous tubules of the animals in the control group. Degenerate seminiferous tubules, with loss of Sertoli cells in animals from the period of exposure to 10 mg in B, which remained degenerate in the animals that passed through the recovery period in E. Desquamation of the semiferiferous epithelium in animals from the period of exposure to 150 mg in C and cells of the seminiferous epithelium deposited in the lumen of the seminiferous tubule in animals that passed through the recovery period in F. ES, seminiferous epithelium; L, lumen of the seminiferous tubule; I, interstice; vs, blood vessel; * loss of seminiferous epithelium. G, H, I germ cells anchored to Sertoli cells (arrow). HE.

Table 1
Matrices for the immediate period.

		Experimental groups		
	Variables	C	LD	HD
Antioxidant system	NP-SH	1.3[a] 1.3 - 1.4	1.3[a] 1.2 - 1.5	1.1[b] 1.0 - 1.2
	GPx	92.9 86.2 - 100.3	102.0 67.7 - 112.8	115.2 100.5 - 119.2
	GST	35.0 31.0 - 35.8	26.9 24.3 - 32.9	26.8 25.0 - 29.9
	LPO	28.1 21.5 - 28.4	27.2 25.0 - 28.6	24.9 17.3 - 29.9
Hormonal influence	Plasma testosterone	233.7 177.8 - 475.5	395.2 386.5 - 456.5	553.1 319.7 - 1019.3
	Intratesticular testosterone	88.0 70.7 - 153.3	151.7 138.0 - 182.9	225.4 189.4 - 304.9
	Testosterone production in vitro	110.6[b] 71.8 - 124.2	130.6[ab] 121.5 - 138.6	269.6[a] 209.5 - 340.2
	Sertoli cells number	22.9[a] 21.8 - 23.2	19.1[b] 17.6 - 19.5	18.6[b] 18.4 - 20.0
	Leydig cells number	73.4 70.0 - 74.2	62.4 59.0 - 76.4	65.3 64.7 - 73.2
Spermatogenesis kinetic	I-VI	28.0[b] 23.0 - 29.0	42.0[a] 41.0 - 43.0	42.0[a] 40.0 - 44.0
	VII-VIII	43.0[a] 39.0 - 46.0	24.0[b] 20.0 - 24.0	26.0[b] 22.0 - 28.0
	IX-XIII	24.0 23.0 - 34.0	30.0 23.0 - 32.0	30.0 28.0 - 30.0
	XIV	5.0 4.0 - 5.0	5.0 3.0 - 6.0	4.0 2.0 - 4.0
	Lumen	17.3[b] 16.7 - 17.6	21.0[a] 19.8 - 22.4	19.2[b] 16.8 - 19.3
	Stroma	12.3[b] 12.1 - 12.7	16.2[a] 16.1 - 17.7	16.1[a] 16.1 - 16.7
	Epithelium	70.5[a] 70.4 - 71.2	61.6[c] 61.3 - 64.2	65.5[b] 64.6 - 67.0
	Number of sperm per g of testis	95.2[a] 93.9 - 96.5	63.1[b] 57.8 - 66.4	71.1[b] 58.0 - 85.2
	Daily sperm production (x10^6 / testis / day)	20.7[a] 19.0 - 21.4	14.0[b] 13.4 - 14.1	14.1[b] 13.1 - 14.7

Data are presented as the median, 1° and 3° quartilhes, One-way ANOVA test with a post test of Tukey or median, 1 and 3 ° quartilhes, *Kruskal-Wallis test with post hoc Dunn's test. $p < 0.05$. C - rats that received only water. LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

Table 2
Matrices for the recovery period.

		Experimental groups		
	Variables	CR	LDR	HDR
Antioxidant system	NP-SH	0.9 0.7 - 1.1	0.7 0.6 - 0.8	0.7 0.7 - 0.8
	GPx	110.9 - 129.8	141.8 130.0 - 170.5	114.5 108.8 - 129.7
	GST	28.6 23.9 - 31.6	24.6 23.2 - 25.4	27.5 22.7 - 30.7
	LPO	32.7 25.0 - 42.6	24.8 24.6 - 25.8	15.6 11.8 - 28.6
Hormonal influence	Plasma testosterone	476.9 252.2 - 752.5	214.5 65.4 - 551.4	260.9 208.5 - 301.5
	Intratesticular testosterone	290.9[a] 85.2 - 484.1	59.7[b] 50.6 - 66.5	99.8[ab] 85.0 - 116.2
	Testosterone production in vitro	278.2[a] 108.7 - 493.0	56.6[b] 52.1 - 76.7	96.4[ab] 84.0 - 112.2
	Sertoli cells number	21.6[a] 20.8 - 22.9	17.6[c] 17.1 - 18.9	19.9[b] 19.2 - 20.9
	Leydig cells number	65.6 57.9 - 66.1	56.4 49.1 - 56.8	68.3 56.8 -70.9
Spermatogenesis kinetic	I-VI	24.0[b] 24.0 - 27.0	44.0[a] 43.0 - 45.0	44.0[a] 39.0 - 44.0
	VII-VIII	44.0[a] 42.0 - 44.0	26.0[b] 24.0 - 26.0	27.0[b] 26.0 - 27.0
	IX-XIII	29.0 28.0 - 29.0	27.0 27.0 - 28.0	26.0 24.0 - 29.0
	XIV	3.0 3.0 - 4.0	3.0 3.0 - 3.0	4.0 3.0 - 4.0
	Lumen	18.3 17.4 - 20.5	19.3 17.7 - 20.6	19.8 19.4 - 20.9
	Stroma	13.5 13.4 - 14.0	13.9 12.2 - 16.1	13.6 13.5 - 14.3
	Epithelium	68.3 66.1 - 70.0	68.5 61.8 - 68.5	66.3 65.8 - 67.1
	Number of sperm per g of testis	88.1[a] 86.0 - 91.7	65.6[b] 63.5 - 69.7	72.0[b] 61.8 - 73.1
	Daily sperm production ($x10^6$ / testis / day)	19.9[a] 19.5 - 20.9	16.0[b] 15.2 - 16.8	16.7[b] 16.1 - 16.9

Data are presented as the median, 1° and 3° quartilhes, One-way ANOVA test with a post test of Tukey. $p < 0.05$. CR – rats that received only water and went through the recovery period; LDR - rats treated with 10 mg/kg of cyantraniliprole and went through the recovery period; HDR - rats treated with 150 mg/kg cyantraniliprole and went through the recovery period.

Table 3

Effects of different doses of cyantraniliprole on immediate and recovery periods on sperm acrosome morphology and integrity

Parameters	Experimental groups Control (n=06 animals)	 10mg (n=06 animals)	 150mg (n=06 animals)
Immediate period			
Number of normal sperm	156[a] 148 - 158	69[b] 60 - 89	55[c] 46 – 58
Number of heads without curvature	6[a] 3 - 7	22[b] 19 - 28	36[c] 33 – 41
Number of isolated heads	7[a] 5 - 7	19[b] 16 - 23	23[b] 20 - 32
Number of isolated tails	10[a] 5 -13	27[b] 22 - 28	26[b] 23 – 36
Number of broken tails	19[a] 14 - 22	33[b] 27 - 40	28[a,b] 21 – 37
Number of curled tails	8[a] 7 - 9	25[b] 18 - 29	28[b] 24 – 34
Number of integral acrosomes	175[a] 156 - 178	63[b] 57 - 67	74[b] 63 – 83
Number of non-integral acrosomes	26[a] 22 - 44	138[b] 133 - 143	127[b] 117 - 137
Recovery period			
Number of normal sperm	141[a] 137 - 147	90[b] 71 - 93	97[b] 88 – 99
Number of heads without curvature	11[a] 10 -12	14[a] 11 - 17	14[b] 13 – 17
*Number of isolated heads	13[a] 11 -14	21[a] 18 - 23	21[b] 17 – 28
Number of isolated tails	8[a] 6 - 12	30[b] 26 - 38	23[c] 19 – 26
Number of broken tails	12[a] 10 - 14	33[b] 27 - 39	26[b] 23 – 28
Number of curled tails	13[a] 12 - 15	16[a] 14 - 19	21[b] 19 – 23
Number of integral acrosomes	143[a] 138 - 147	58[b] 43 - 65	63[b] 56 – 67
Number of non-integral acrosomes	58[a] 53 - 62	143[b] 135 - 157	138[b] 133 - 144

Data are presented as the median, 1° e 3° quartilhes. One-way ANOVA test with a post test of Tukey or *Kruskal-Wallis test with post hoc Dunn's test. $p < 0.05$. LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

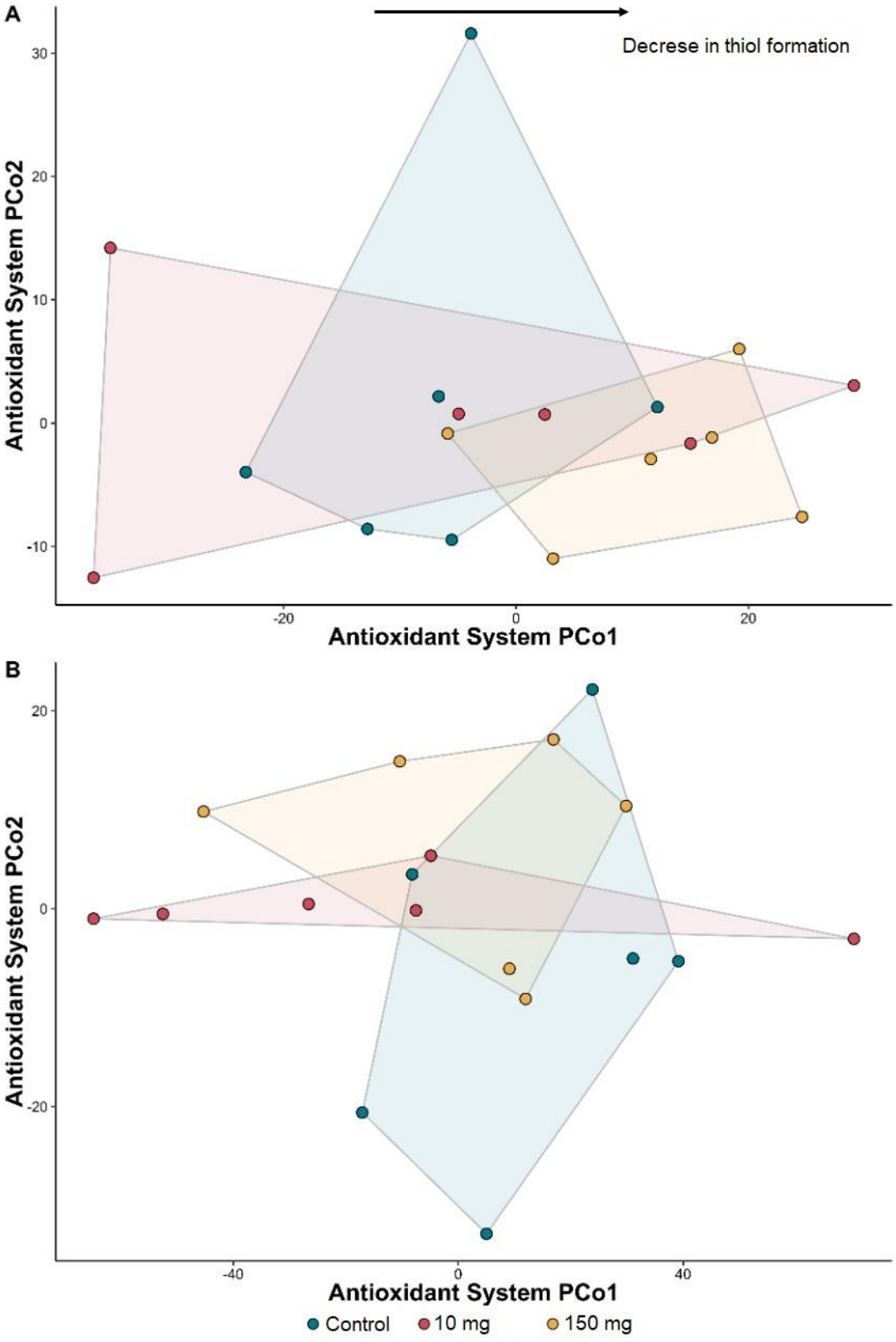
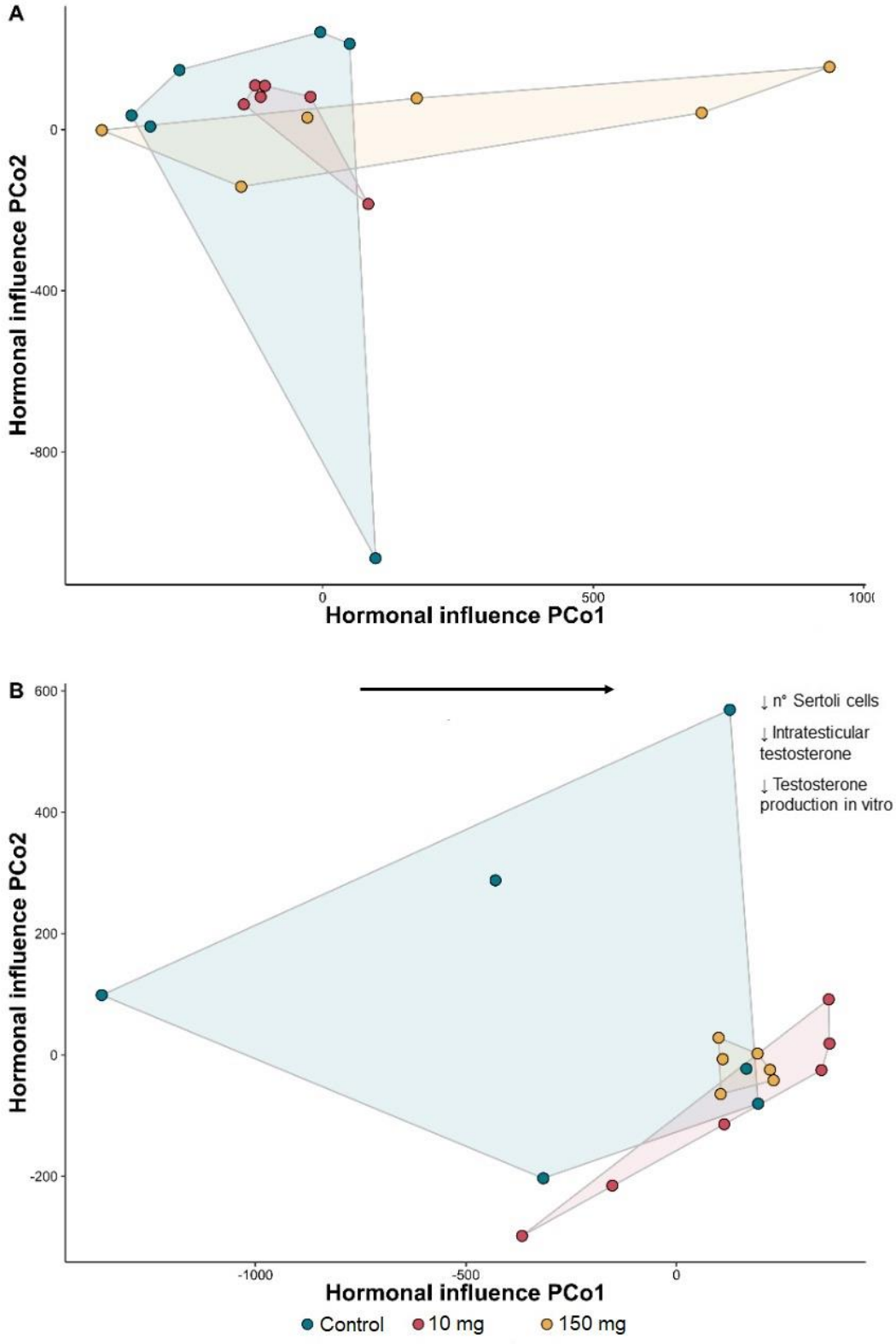


Figure 1. Principal Coordinates Coordinates Analysis (PCoA) plot of testicle Antioxidant System matrices: A: Immediate period. B: Recuperation period.



1

2 Figure 2. Principal Coordinates Coordinates Analysis (PCoA) plot of hormonal

3 influence matrices: A: Immediate period. B: Recuperation period.

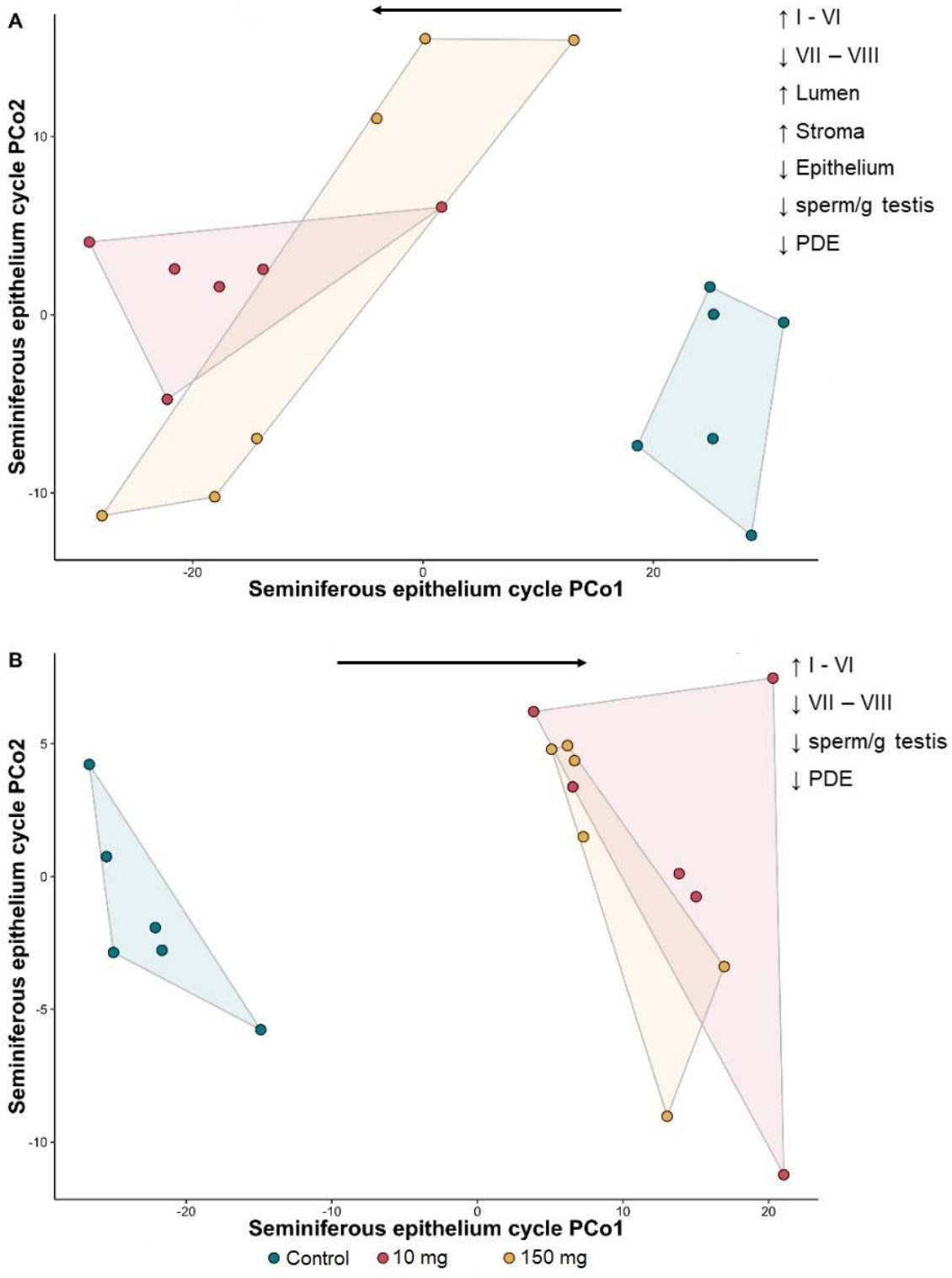


Figure 3. Principal Coordinates Coordinates Analysis (PCoA) plot of Seminiferous epithelium cycle matrices: A: Immediate period. B: Recuperation period.

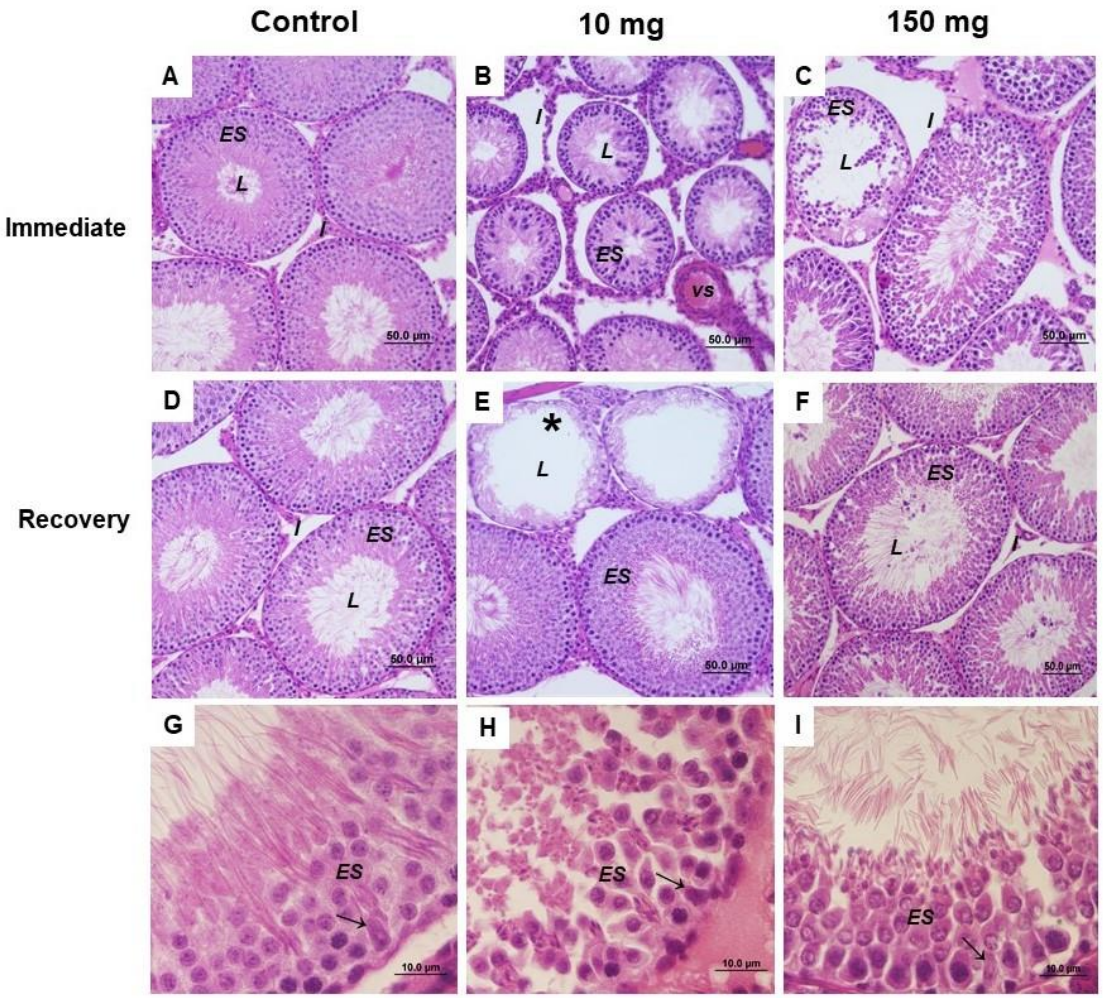


Figure 4. Histopathological analysis of the testis. In A and D, normal characteristics of the seminiferous tubules of the animals in the control group. Degenerate seminiferous tubules, with loss of Sertoli cells in animals from the LD in B, which remained degenerate in the animals that passed through the LDR in E. Desquamation of the seminiferous epithelium in animals from the HD in C and cells of the seminiferous epithelium deposited in the lumen of the seminiferous tubule in animals HDR in F. *ES*, seminiferous epithelium; *L*, lumen of the seminiferous tubule; *I*, interstice; *vs*, blood vessel; * loss of seminiferous epithelium. G, H, I germ cells anchored to Sertoli cells (arrow). HE.

ARTIGO 3

ADULT FEMALE EXPOSURE TO CYANTRANILIPROLE IMPAIRS REPRODUCTIVE PARAMETERS BY INDUCING OXIDATIVE STRESS IN WISTAR RATS

Artigo será submetido à revista — “Reproductive Toxicology”.
ISSN: 0890-6238;
F.I. 2019: 3.1
Qualis CAPES 2013 - 2016 (Medicina II): A.2

ADULT FEMALE EXPOSURE TO CYANTRANILIPROLE IMPAIRS REPRODUCTIVE PARAMETERS BY INDUCING OXIDATIVE STRESS IN WISTAR RATS

Suellen Ribeiro da Silva Scarton[1,2], Felipe Tsuzuki[1], Marina Trevizan Guerra[3], Dayane Priscila dos Santos[1,2], Aldair Casagrande dos Santos[4], Ana Tereza Bittencourt Guimarães[4], Andréa Name Colado Simão[2], Célia Cristina Leme Beu[4], Glaura Scantamburlo Alves Fernades[1*]

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

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

[3]Federal University of Mato Grosso do Sul, Campus Três Lagoas, 3484 Ranulpho Marques Leal Avenue, Postal code: 79613-000, Três Lagoas, Mato Grosso do Sul, Brazil

[4]Medical and Pharmaceutical Sciences Center, State University of Western Paraná - UNIOESTE, Universitária street, 1619, PR, Postal code: 85819-110, Cascavel, Paraná, Brazil

*Corresponding author

Email address: glaura@uel.br

Abstract

This study aimed to verify the effects of exposing adult female Wistar rats (67 days old) to doses of 10, 150 mg/kg of cyantraniliprole, orally, for 28 days. The control group received water through the same route. For 28 days, vaginal washings were performed to assess the regularity of the estrous cycle. Euthanasia occurred in DPN95, blood was collected, for progesterone dosage, uterus, and ovaries for morphometric and histopathological evaluations, ovarian follicle count, and biochemical markers of oxidative stress. Liver, kidneys, pituitary, adrenal gland collected. all organs were weighed. The progesterone concentration and the weight of the uterus, ovary, liver, kidneys, adrenal gland and a were higher at 10 mg/kg. The number of estrous cycles was decreased and the cycle duration increased by 150 mg/kg. The glandular epithelium of the uterus was larger and the number of ovarian follicles, primordial+primary, preantral, antral, and atretic were reduced by 10 mg/kg. Histopathological analysis revealed the occurrence of metaplasia of the luminal epithelium of the uterus with the presence of mitosis and polymorphism of the glandular epithelium cells, in addition to the presence of inflammatory infiltrate in the endometrium, in the ovaries, congested blood vessels, hyperplasia of the rete ovarii were observed. The activity of cholinesterase in the uterus was lower at both doses, higher occurrence of lipoperoxidation and SOD enzyme activity occurred only at 10 mg/kg, however, the GST activity was higher at 10 and 150 mg/kg. In the ovary, cholinesterase activity was increased in animals treated with both doses, and GPx activity was higher only at 10 mg/kg. With the results obtained, we conclude that cyantraniliprole exerts negative effects on the female genital tract, mainly at low doses.

Keywords: insecticide, reproduction, uterus, ovaries.

1. Introduction

Currently, Brazil leads the use of substances for pest control in agricultural production [1]. Understanding the possible interactions between biological systems and chemical compounds used in farming processes can determine more sustainable practices for the environment and consequently contribute to human health. Cyantraniliprole is an insecticide in the class of anthranilic diamides, capable of modulating calcium channels known as Ryanodine receptors (RyRs). Insects express a single form of this receptor, while mammals express three isoforms, RyR1 and RyR2, distributed predominantly in the skeletal and cardiac muscle, respectively, and RyR3 heterogeneously distributed in the muscle tissues, with the homology of the receptors between these species being 47% [2]. The binding of cyantraniliprole to RyRs causes the unregulated release of calcium (Ca^{2+}) from the sarcoplasmic reticulum and, consequently, the breakdown of the intracellular homeostasis of this ion [3,4]. Besides, studies have shown the presence of RyRs in the reproductive tissues such uterus from rats [5] and humans [6], and ovaries (DIAZ-MUNOZ *et al.,* 2008).

The control of intracellular Ca^{2+} plays an important role in multiple biological processes, including cell signaling, muscle contraction, release of neurotransmitters, and fertilization [7], and thus, the Ca^{2+} signaling pathways interact with other cellular signaling systems, as that exerted by reactive oxygen species (ROS) [8]. The interactions between ROS and Ca^{2+} are considered bidirectional, as ROS can regulate the Ca^{2+} signaling function, while Ca^{2+} signaling is essential for the formation of ROS [9]. Although, in a healthy organism, ROS and antioxidant systems remain in balance, the breakdown of this homeostasis generates an increase in the production of ROS and as a consequence, oxidative stress (OS) [10]. The pathological effects of OS are

exerted by several mechanisms that include lipid damage, inhibition of protein synthesis, and depletion of ATP [11].

ROS are pro-oxidants and in the female genital system they may act as key signaling molecules in physiological processes or, contrary, in pathological processes that involve the female reproductive tract [12]. The physiological effects of ROS involve the maturation of oocytes, steroidogenesis [13–15], and maintenance of corpus luteum cells in the occurrence of pregnancy [16]. On the other hand, OS is involved in the pathophysiology of pre-eclampsia [17,18], hydatidiform mole [19], ovarian cancer [20], polycystic ovary syndrome [21] endometriosis [22,23], the occurrence of spontaneous abortions and recurrent pregnancy losses [24].

The excess production of ROS and the subsequent induction of OS, are known to negatively affect reproductive functions [12], with chemical substances and pollutants being the potential initiators of these processes, which may culminate in reproductive disorders and infertility [25]. It is known that several agrochemicals are considered as endocrine disruptors [26,27]. Endocrine disrupting chemicals are able to interfere with the synthesis, secretion, transport, activity, or elimination of natural hormones and this interference can block or mimic hormone action, causing a wide range of negative effects [28]. The mass production of chemicals used in crops and its wide distribution and presence in several consumer goods, lead to daily human exposure [29], representing a threat to the health of the general population [30]. However, due to the recent use of cyantraniliprole in crop cultures, to date, there are no studies reporting the effect of this insecticide on the female genital tract of humans or rodents. Thus, this study aimed to evaluate the possible effects of cyantraniliprole on the uterus and ovaries of adult Wistar rats.

2. Material and methods

2.1. Animals and experimental conditions

Adult female Wistar rats (n=24, 60 days of age), were obtained from the Central Vivarium of the State University of Londrina - UEL, allocated in the sectoral vivarium of the Department of Physiological Sciences - UEL, in polypropylene cages (43 x 30 x 15) (4 animals per cage) and maintained in a 12 h light / dark cycle, 22 °C, water and standard feed *ad libitum*. Animal care and handling procedures were conducted following the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised in 1978) and with the approval of the Ethics in Use Committee of Animals of the State University of Londrina (OF. CIRC. CEUA nº134 / 2017, process n ° 21106.2017.24).

2.2. Experimental design

After one week of acclimation, females (67 days of age) were distributed in the following experimental groups (n = 8 animals/group): control group (C, which received tap water - vehicle), and two groups exposed to 3-bromo-1-(3-chloro-2-pyridyl)-4′-cyano-2′-methyl-6′-(methylcarbamoyl) pyrazole-5-carboxanilide - Cyantranilipore (Benevia® 10%m/v - Du Pont Brasil S.A., Barueri, SP, Brazil) at dose of 10 mg/kg (low dose - LD) or to 150 mg/kg (high dose - HD). Once that nowadays there is no knowledge on the reproductive toxicity of this chemical available in the literature, the doses used in this present experiment were based on the chemical´s Safety Data Sheet information (Safety Data Sheet, 2021).

All animals were treated orally (gavage) for 4 consecutive weeks (28 days), comprising at least 05 complete estrous cycles [31]. Body weight, water, and food consumption were measured twice a week. At the end of the treatment, during the first estrus after PND 95, all animals were weighted, anesthetized with inhalation anesthetic saturation with Isoforine® 1.28% (Cristália, Itapira, SP, BR) and euthanized by exsanguination.

2.3. Preparation of Cyantraniliprole

Cyantraniliprole (3-Bromo-1- (3-chloro-2-pyridinyl) -N- [4-cyano-2-methyl-6 - [(methylamino) carbonyl] phenyl] -1 H-pyrazole-5-carboxamide; 10.0% w/v), was received as a donation from the Center for Agricultural Sciences of the State University of Londrina - UEL. Tap water was used as a vehicle for the dilution of the toxic agent.

2.4. Analysis of the estrous cycle

The evaluation of the regularity of the estrous cycle was carried out during the whole treatment (28 days, approximately 05 complete cycles). Vaginal lavages were obtained with the aid of a pipette containing 10 µL of 0.9% saline solution; then, the liquid containing the desquamated cells of the vagina was distended on a histological slide and analyzed under a 40x objective light microscope. According to the predominant cell types, the phase of the estrous cycle was characterized in proestrus (predominance of nucleated epithelial cells), estrus (predominance of cornified epithelial cells), metaestrus (presence of cornified epithelial cells and leukocytes), or diestrus (predominance of leukocytes) [31]. The data were used to calculate the frequency of each cycle phase (%), the number of estrous cycles (days), and the average duration of each cycle (days).

2.5. Plasma progesterone level

Blood was collected from the left renal artery, using a previously heparinized syringe, subjected to centrifugation at 10,000 g for 10 minutes. The progesterone present in the plasma was measured via Architect progesterone (Abbott, Wiesbaden, Germany), according to the manufacturer’s recommendations. The intra-assay

coefficient of variation and minimum sensitivity of the assay was 6.2% and 0.1 ng/mL respectively.

2.6. Organs collection

Following the euthanasia on estrus phase, detoxifying organs as liver and kidneys, reproductive organs (uterus and ovaries), right adrenal gland and pituitary were collected and weighed (n = 6 females per group). The middle portion of the right uterine horn and the right ovary were fixed in Davidson's modified fluid (30% of 40% formalin solution, 15% ethanol, 5% glacial acetic acid and 50% distilled water) for 06 hours, post-fixed in 4% paraformaldehyde for 18 hours, dehydrated in an alcoholic series and included in Paraplast® (Sigma Aldrich). The material was sectioned at 3 μm (03 sections per animal, with an interval of 30 μm among them), and stained with hematoxylin and eosin (HE) to perform morphological analyzes. All histological sections of the uterus and ovary were photocumented with the aid of an Olympus DP71 microscope attached to the Olympus BX60® camera (Olympus Corporation, Tokyo, Japan). The generated data were used in the statistical analyses.

2.6.1. Morphometry and histopathological analysis of the uterus

In the uterus, the morphometry of the myometrium, endometrium and glandular and luminal epithelium were performed. Five different regions, from each one of the three sections, were evaluated, making a total of 15 measurements per animal [32]. Myometrium and endometrium were evaluated in 200-fold magnification, glandular and luminal epithelium in 400-fold magnification. Histopathological analysis was performed from the same images to identify the presence of metaplasia, formation of cysts, and endometrial hyperplasia.

2.6.2. Ovarian follicle and corpora lutea count

In the right ovary, the ovarian follicles and the corpus luteum were counted and then the average of the three sections of each animal was performed. The follicles were classified according to [33,34]. Primordial and primary follicles were counted together, and all oocytes showing a single layer of squamous or cuboidal epithelial cells were contacted. The follicle was classified as pre-antral by identifying 2-4 layers of granulosa cells without the presence of the antral space. Antral follicles were classified based on the visualization of 3 or more layers of granulosa cells and well-defined antral space. Atretic follicles were identified by the presence of pycnotic nuclei and oocyte degeneration and disorganization of granulosa cells.

2.7. Cholinesterase (ChE) activity

The analysis of ChE activity was performed using the method published by Ellman *et al.*, (1961) and modified for microplate. The principle of the method is to measure the production of thiocoline when acetylthiocholine (ATC) is hydrolyzed, which is accomplished by continuously reacting the thiol with the 5, 5 'dithiobis-2-nitrobenzoic acid (DTNB) to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The reaction was carried out in triplicate in 300 µL of the solution containing 0.05 DTNB and 1.5 mM ATC. ChE activity in relation to protein concentration (mg.mL-1) was calculated using the molar extinction coefficient of DTNB (1.36mM-1.cm-1). The results were expressed in nmol.min-1. mg of protein-1.

2.8. Biomarkers of oxidative stress

The middle portion of the left uterine horn and the left ovary were homogenized in 2 mL of phosphate buffer (pH 7.4) and centrifuged at 9,500 g for 10 min at 4ºC. The

protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as a standard [36]. The supernatant was separated and used for the following analyzes.

2.8.1. Lipid peroxidation - LPO

Lipoperoxidation was measured to indirectly quantify the peroxides produced. The result reflects the intensity of lipid peroxidation [37]. The measurements were performed using the method of reactive substances to thiobarbituric acid (TBARS) with an absorbance of 535 nm [38] and with comparison to the standard curve for malondialdehyde (MDA), the main by-product of cellular lipid peroxidation. To prepare the test, a 0.33 mg.mL-1 aliquot of the sample protein was added in 6.7% trichloroacetic acid (TCA) in a final volume of 180 µL and centrifuged for 5 min at 13,860 g at 4ºC. For the measurement of substances reactive to thiobarbituric acid (TBARS), the following substances were added to the microplate: 40 uL of the supernatant and different concentrations of MDA in triplicate, and reaction medium containing 21.42 mM of thiobarbituric acid (TBA), 17, 86 mM NaOH (used for TBA solubilization), 0.73 M TCA, 0.032 mM butylated hydroxytoluene (BHT) and 3% ethanol (used for BHT solubilization) in PBS buffer. Lipid peroxidation was estimated from the MDA curve, and the results are expressed in nmol of TBARS. mg of protein-1.

2.8.2. Glutathione transferase (GST) activity

This analysis assesses the enzymatic activity of GST in catalyzing the conjugation of GSH with the synthetic substrate CDNB, which produces a conjugate detected at 340 nm [39]. During the assay, the enzyme activity is proportional to the production rate of the conjugated compound. The assay was performed in triplicate, in

a 96-well microplate, and the final concentration of the sample was 0.020 mg of protein.mL-1. The reaction medium produced final concentrations of 0.94 mM CDNB and 0.94 mM GSH. The molar extinction coefficient of the compound GSH/CDNB was 9.6 mM-1.cm-1, and the unit was expressed as mmol.min-1.mg of protein-1.

2.8.3. Superoxide dismutase (SOD) activity

SOD activity was assessed according to the method originally proposed by Crouch *et al.*, (1981) with the modifications described below. The principle of this analysis was to quantify the complex formed between superoxide and nitrotetrazolium blue (NBT), measured at 560 nm for 1.5 hours. An aliquot of 0.75 mg.mL-1 of protein in 25% ethanol was prepared in a total volume of 800 µL. The sample was centrifuged at 13,680 g at 4ºC for 20 minutes. The supernatant was pipetted in triplicate, in a 96-well microplate; the final 200µL volume contained: 0.1 mg protein.mL-1, 0.09 mM NBT, 0.015 mM EDTA, 34.78 mM hydroxylamine sulfate and 79 mM sodium carbonate buffer (pH 10, two). The activity values of the antioxidant enzymes were expressed in U. mg of protein-1. A unit of SOD activity was defined as the amount of enzyme that inhibits the oxidation reaction of NBT by 50% of maximum inhibition.

2.8.4. Glutathione peroxidase (GPx) activity

GPx activity was evaluated according to the technique proposed by (Flohé & Günzler, (1984). The enzymatic activity was accompanied by a decrease in the absorbance of NADPH at 340 nm. The reaction system was composed of phosphate buffer 100 mmol.L-1 (pH 7.0) EDTA 1 mmol.L-1, GSH 2 mmol.L-1, NADPH 0.15 mmol.L-1, purified glutathione reductase 0.2U, 0.5 mmol.L-1 t-butyl hydroperoxide.

The reaction was initiated by the addition of t-butyl hydroperoxide and monitored for 1 minute. GPx was expressed in µmoles of oxidized NADPH. min-1. mg of protein-1.

2.8.5. Glutathione reductase (GR) activity

GR catalyzes the reduction of glutathione disulfide (GSSG) through the oxidation of NADPH; the decrease in absorbance was measured at 340 nm [42]. The assay was performed in duplicate, in a 96-well microplate. The final concentrations in the reaction medium were: 0.138 mM NADPH, 3.81 mM GSSG and 3.75 mM EDTA. The molar extinction coefficient of NADPH was 6.22 mM-1.cm-1. The unit was expressed in mmol.min-1.mg of protein-1.

2.9. Statistical analysis

The Shapiro-Wilk test was used to verify the normal distribution of the data, and the homogeneity of the variance between the groups was assessed by the Levene test. One-way analysis of variance (ANOVA) with Tukey post hoc test or Kruskal-Wallis non-parametric test with Dunn's post hoc test was used to compare the results among experimental groups. Differences were considered significant when $p < 0.05$. Data are presented in mean ± S.E.M., when parametric or median (1º and 3 ° quartiles), when non-parametric. Statistical analyzes and graphs were performed using the IBM® SPSS® Statistics program (IBM Corp. Launched in 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

3. Results

3.1. Body and organ weights

The body weight (initial and final) and organs weight are shown in table 1. No statistical differences were observed in the initial and final body weight among experimental groups. However, the uterus weight was increased in animals exposed to a high dose of cyantraniliprole, when compared to control group. The animals from LD group presented higher ovarian and adrenal weight when compared to C animals. Exposure to both doses caused an increase in liver weight compared to control. The assessment of the weight of the pituitary gland showed no statistical difference among the experimental groups.

Table 1. Effects of exposure to different doses of cyantraniprole for 28 consecutive days, during adulthood, at estrus phase, on female body weight and organs weights.

Parameters	**Experimental groups**		
	Control (n=06 animals)	LD (n=06 animals)	HD (n=06 animals)
Body weight			
[1] Initial (g)	178,0 ± 4,0	188,0 ± 8,0	181,0 ± 9,0
[1] Final (g)	221,0 ± 9,0	232,0 ± 7,0	241,0 ± 10,0
[2] Uterus (mg)	421,0 (395,0 – 506,0) [a]	533,0 (469,0 – 607,0) [a,b]	755,0 (513,0 – 904,0) [b]
[1] Right ovary (mg)	32,0 ± 1,1 [a]	39,3 ± 1,1 [b]	35,3 ± 2,2 [a, b]
[2] Liver (g)	6,8 (6,0 – 7,5) [a]	8,7 (8,7 – 9,2) [b]	9,0 (8,2 – 10) [b]
[1] Kidneys (mg)	761,6 ± 38,3 [a]	824 ± 16,9 [b]	714,8 ± 22,6 [b]
[2] Adrenal (mg)	38,5 (32,0 – 42,0) [a]	53,0 (38,0 – 74,0) [b]	37,5 (36,0 – 41,0) [a,b]
[1] Pituitary (mg)	25,5 ± 14,8	13,3 ± 10,0	19,7 ± 8,6

[1]Mean ± S.E.M, One-way ANOVA test with a post test of Tukey.

[2]Median (1º and 3 ° quartiles), Kruskal-Wallis test with post hoc Dunn's test.

Control - rats that received tap water; LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

Different letters mean significate difference, with $p < 0.05$.

3.2. Analysis of the estrous cycle

The analysis of the regularity of the estrous cycle revealed that cyantranilirole was not able to disrupt the frequency of each phase of the estrous, in neither low or high doses. However, it was observed, in the HD group, an increase in the duration of the estrous cycles (in days) and a consequent decrease in the number of cycles when compared to the LD and C groups (Table 2).

3.3. Plasma progesterone level

The level of progesterone was significantly increased in the group that received the low dose of cyantraniliprole compared to the control and HD (Table 2).

Table 2. Frequency (%) and duration (days) of the estrous cycle during the period of exposure to Cyantraniliprole (28 consecutive days) and hormonal concentration, at the endo of the exposure period, from female animals, at adulthood, in estrus phase.

Parameters	**Experimental groups (n=06 animals/group)**		
	Control	LD	HD
[1]Estrous phases frequency (%)			
Proestrus	20,97 (15,35 – 22,58)	14,52 (12,10 – 19,35)	19,35 (18,55 – 23,39)
Estrus	32,26 (29,03 – 41,93)	27,42 (25,00 – 29,84)	24,20 (18,55 – 33,06)
Metaestrus	16,13 (16,13 – 20,16)	19,35 (15,32 – 19,35)	14,55 (9,68 – 23,39)
Diestrus	27,43 (21,78 – 35,48)	41,94 (33,88 – 42,75)	40,33 (33,87 – 46,78)
[2]Number of estrous cycles	6,25 ± 0,67[a]	6,25 ± 0,25[a]	4,50 ± 0,50[b]
[2]Estrous cycle length (days)	4,24 ± 0,35[a]	4,51 ± 0,52[a]	6,16 ± 1,36[b]
[1]Progesterone ng/mL	10,78 ± 5,22[a]	24,83 ± 9,84[b]	12,28 ± 4,13[a]

[1]Median (1º and 3° quartiles), Kruskal-Wallis test with post hoc Dunn's test.

[2]Mean ± S.E.M, One-way ANOVA test with a post test of Tukey.

Control - rats that received tap water; LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

Different letters indicated statistical difference, with $p<0.05$.

3.4. Morphological analysis of the reproductive organs

The results from reproductive organs analysis are shown in table 3. There were no statistical differences in the height of the myometrium, endometrium, or luminal epithelium between animals that received cyantraniliprole when compared to the control group. The animals from HD group showed a reduced glandular epithelium height when compared to the other experimental animals. Histopathological analysis of the uterus from female animals exposed to cyantraniliprole for 28 consecutive days revealed areas with the presence of mitotic cells in the luminal epithelium, epithelial metaplasia (fig. 1B and C) and glandular epithelial polymorphism, desquamated cells in glandular lumen and inflammatory infiltrate in the endometrial stroma (fig. 1E and F).

Regarding ovarian follicles assessment, a significant decrease was observed in the number of primordial+primary follicles in the group exposed to the high dose when compared to the control group. Both LD and HD groups demonstrated significantly reduction in the number of preantral, antral, and atretic follicles when compared to C animals. The number of corpus luteum was not affected by cyantraniliprole exposure (Table 3). The histopathological analysis of the ovary showed that both doses caused damage to the tissue, observed by areas with congested blood vessels in the medular region, in addition to the deflation of the ovarii rete (fig. 2).

Table 3. Effects of exposure to different doses of cyantraniprole for 28 consecutive days, on reproductive organs, from female adult Wistar rats, in estrus phase.

Parameters	Experimental groups (05 animals/group)		
	C	LD	HD
[1]Uterine morphometry (μm)			
Myometrium	184,54 ± 16,10	181,97 ± 10,20	242,23 ± 26,67
Endometrium	339,93 ± 12,68	396,52 ± 6,86	361,31 ± 28,25
Luminal epithelium	17,88 ± 2,99	19,08 ± 2,42	19,05 ± 1,77
Glandular epithelium	7,66 ± 0,68 [a]	8,76 ± 0,44 [a]	5,65 ± 0,23 [b]
Ovarian follicles count			
[1]Primordial + primary	16,66 ± 1,98[a]	12,13 ± 1,04 [a,b]	9,53 ± 1,14 [b]
[2]Preantral	19,53 ± 1,10 [a]	10,80 ± 0,96 [b]	8,20 ± 0,47 [b]
[1]Antral	15,80 ± 1,46 [a]	11,26 ± 0,83 [b]	10,80 ± 1,01 [b]
[2]Atretic	7,33 (5,00 – 7,00) [a]	1,66 (1,66 – 3,00) [b]	2,33 (1,66 – 2,6) [b]
[2]Corpus luteum number	5,86 ± 0,70	6,13 ± 0,58	6,40 ± 0,28

Data are presented as the [1]mean ± s.e.m, One-way ANOVA test with a post test of Tukey or [2]median, 1 and 3 ° quartilhes, Kruskal-Wallis test with post hoc Dunn's test. $p < 0.05$. C - rats that received tap water; LD - rats treated with 10 mg/kg of cyantraniliprole; HD - rats treated with 150 mg/kg cyantraniliprole.

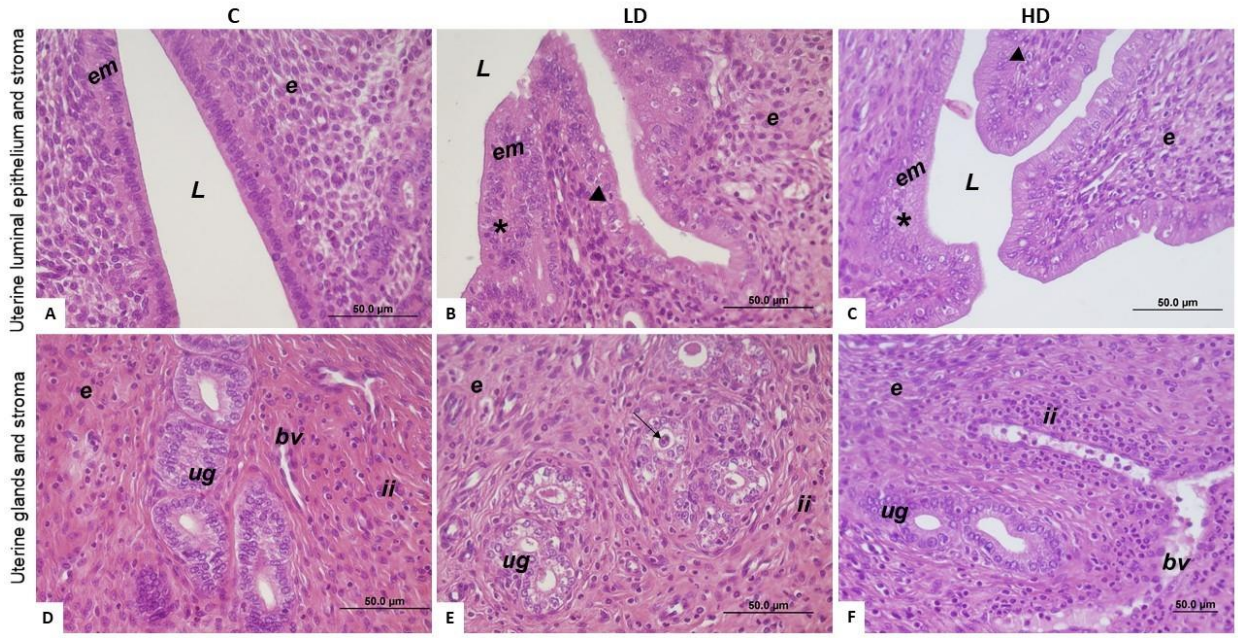


Fig. 1. Histopathology of the transversal section from medial region of the uterine horn, at estrus phase, from animals exposed to the vehicle (C), 10 mg/kg (LD) or 150 mg/kg (HD) of cyantraniliprole, at adulthood. Control animals reveals normal appearance of luminal **(A)** and glandular **(D)** epithelium, and normal stroma of the uterine mucosa **(A and D)**; Animals from LD group presented metaplasia of the luminal epithelium, mitotic cells **(B)**, glandular epithelial polymorphism, desquamated cells in glandular lumen and inflammatory infiltrate **(E)**; HD group showed metaplasia of the luminal epithelium, mitotic cells **(C)**, massive inflammatory infiltrate **(F). *e*:** endometrial stroma; *L*: lumen; *em*: endometrial epithelium; *bv*: blood vessel; *ug*: uterine glands; *ii*: inflammatory infiltrate; asterisk: metaplasia; arrowhead: mitotic cells; arrow: desquamated cells. HE. (A, B and C: 100X magnification; D, E and F: 400X magnification).

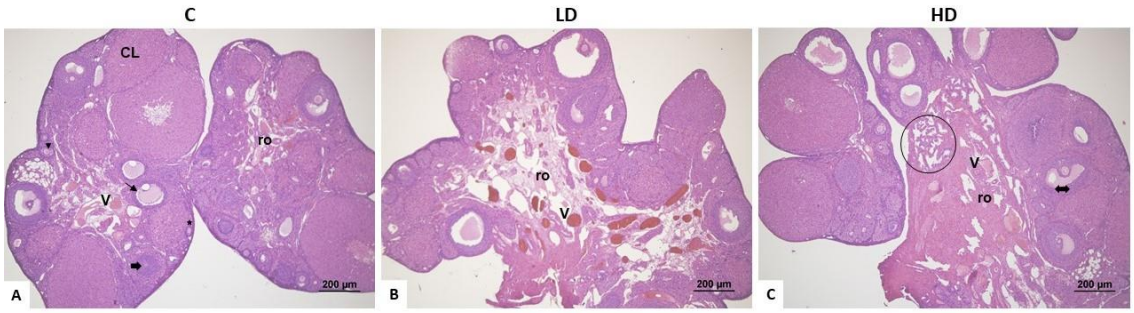


Fig. 2. Histopathology of the ovary of animals, in estrus phase, exposed to the vehicle (C), 10 mg / kg (LD) or 150 mg / kg (HD) of cyantraniliprole from PND67 to PND95. In **(A)**: Control group (C) shows normal aspect of the ovary with the presence of follicles in different phases of folliculogenesis and corpora lutea in the cortical area and rete ovarri with regular architecture; **(B)** and **(C)**: LD and HD groups, demonstrating regular cortical region but with presence of rete ovarii hyperplasia with congested blood vessels; wide arrow: atresic follicle; CL: corpora lutea, and V: blood vessels in the rete ovarii (ro). The circled area shows the formation of clefts lined by a cuboidal to columnar epithelium, compatible with epithelial hyperplasia of the rete ovarri. HE. (40X magnification).

3.5. Biomarkers of oxidative stress

The evaluation of biomarkers of oxidative stress in adult ovary and uterus, after cyantraniliprole exposure at both doses reveals that the activity of ChE was diminished in the uterus (3A) and increased in the ovaries (3G), when compared to control rats. Uterine lipid peroxidation (LPO) was increased in animals from LD group, when compared to control and HD animals (3B), but these values were similar among groups on ovarian tissue (3H). SOD activity assessment demonstrated comparable results in the ovary (3I), but it was higher in the uterus from LD when compared to control (3C). This LD experimental group also presented elevated activity of GPx in the ovary (when compared to the non-exposed and HD animals – 3J), but regarding uterus, this enzyme presented no difference in the activity among groups (3D). GST presented no statistical

difference among groups in the ovary, but it was increased in the uterus after cyantraniliprole exposure (3E). GR level was not altered after the exposure to the insecticide (uterus and ovaries – 3F and 3L).

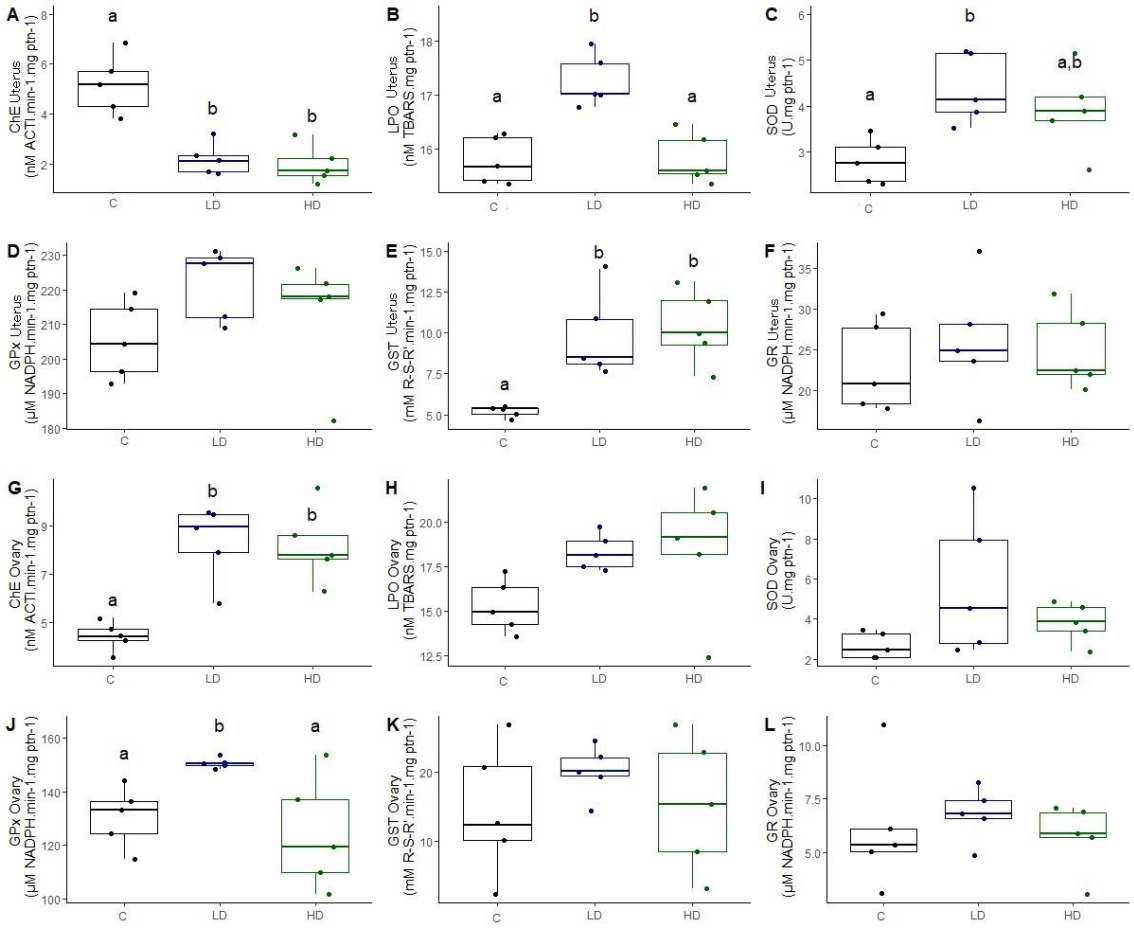


Fig. 3. Assessment of the cholinergic system and biomarkers of oxidative stress in the uterus and ovaries of animals exposed to the vehicle (C) or cyantraniliprole 10 mg/kg (LD) or 150 mg/kg (HD). From A to F evaluations of the uterus and from G to L evaluations of the ovary. ChE, cholinesterase; LPO, lipid peroxidation; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, Glutathione S-transferase; GR, glutathione reductase. Data are presented as the median, 1 and 3 ° quartiles. Kruskal-Wallis test with post hoc Dunn's test. Different letters mean significant difference, with $p < 0.05$.

4.0. Discussion

Many studies have indicated that several pesticides are able to alter the structure and function of the female reproductive tract and organs related to the production and metabolism of hormones [43]. In this study, cyantraniliprole, an insecticide from anthranilic diamides class, was able to disrupt the oxidative system in the uterine and ovarian tissues, increases the weight of the adrenal gland and liver, interferes with the duration of the estrous cycle and elevates the production of progesterone.

The liver possesses an important role in organism detoxification [44] and chronic liver diseases are commonly characterized by increased oxidative stress [45]. We observed significant increased liver weight in both groups of animals exposed to cyantraniliprole for 28 consecutive days, indicating toxicity of this compound to this organ, probably due to OS induction.

In order to evaluate the cholinergic system, the level of ChE activity was measured. In the ovary, granulosa cells are able to produce and store acetylcholine (ACh) [46] and express muscarinic receptors [47], and the presence of acetylcholinesterase (AChE) have been reported in this organ [48]. The presence of Ach is related to increased follicular development [49]. Both experimental groups exposed to cyantraniliprole presented elevated level of ChE activity, which could be related to follicle impairment, observed in the histological analyses. Cholinergic nerves are present in the uterine endometrium and myometrium [50]. Most of the efferent inputs to the uterus that stimulates contractions are cholinergic (MORIZAKI et al. 1989), acting though muscarinic receptors [51] and in the endometrium, it is postulated the role of cholinergic control on the secretory activity [52]. The lower value of ChE in

the uterus (probably due to local elevated ROS production) from exposed females indicates disturbance in the local cholinergic system. [53–56]

Differences in oxidative status between the uterus and ovaries have already been demonstrated and it appears that the ovary exhibits greater protection against the formation of ROS [57]. Maintaining the balance between the production of ROS and antioxidants is important to preserve the normal function of the organ. The adequate amount of ROS is necessary for ovarian cells [10] because it is involved in the growth of ovarian follicles, mainly by regulating angiogenesis [58], participating in oocyte maturation [59] and ovulation [60]. On the other hand, in the uterus, OS can be the cause of recurrent embryo implantation failures and spontaneous abortions [57].

We have demonstrate that exposure to low dose of cyantraniliprole (10mg/kg) is able to induce the generation of OS by increasing lipid peroxidation in reproductive organs. In response to this OS, the activity of GST enzyme in uterus was elevated in order to promote detoxification of lipid peroxidation products [61,62]. In the ovary, despite normal level of LPO, the GPx activity was significantly increased, indicating that lipid peroxidation in this organ was sufficient to strongly induce the expression of this enzyme and stablish an adaptive process to protect the ovary from cyantraniliprole exposure toxicity [62,63].

SOD is an enzyme responsible for $O_2^{\cdot -}$ scavenger, catalyzing the dismutation of superoxide to H_2O_2, and it is present in all subcellular compartments susceptible to oxidative stress [64,65]. Females exposed to the lowest tested dose of cyantraniliprole presented elevated uterine levels of SOD activity, representing another protective mechanism of this organ against ROS.

Salzano *et al.*, (2014) have demonstrated that the OS in the uterus provokes a release of molecules that have been recognized as inflammatory signals; ROS and

oxidative stress are very important in controlling stages of cell proliferation, survival, aging and death [67,68] and it is known that cancer cells present elevated production of reactive oxygen species [69]. The oxygen reactive species production in the uterus promoted the recruitment of defense cells, leading to inflammatory infiltration in uterine stroma, besides alterations on the epithelial cells as luminal metaplasia, glandular hypoplasia and detachment of cells into the glandular lumen.

Physiological levels of ROS regulates foliculogenesis and oocyte maturation [70]. However, exacerbated production of these molecules leads to OS and may induces oocyte and granulosa cells apoptosis [71,72]. The oxidative stress induced by ciantraniliprole exposure caused adverse effects in the ovarian tissue, revealed by depletion of non-selective ovarian follicles, diminished cortical area with enlarged medullar region and blood vessels congestion. Elevated levels of ROS may also disrupt estrous cycle and induce infertility (AGARWAL 2005). This fact could explain the disruption of estrous cyclicity in female animals after 150mg/kg exposure of cyantraniliprole.

The source of the elevated level of progesterone, detected in female rats after 28 consecutive days of exposure to cyantraniliprole at 10mg/kg, could be addressed to the adrenal gland, once that this organ presented to be enlarged in this same group, and the number of corpora lutea in the ovary was not affected. Contrary, previous studies reported that OS decreases adrenal steroidal production by reducing steroidogenic enzymes activity [73,74]. With the methodology applied in this experiment, we were not able to determine the mechanism by which adrenal increases its steroidal production. Therefore, this should be an important issue for futures studies. The increment in progesterone levels could be seen as an attempt to promote

protection against ROS, once that this hormone reduces OS, possible via SOD activity regulation [75,76].

5.0. Conclusion

This study indicated that exposure to cyantraniliprole in female adult at doses of 10 and 150mg/kg, from 28 consecutive days, caused liver toxicity, harmed cholinergic system in the reproductive organs, damaged the ovarian and uterine histological architecture, stimulated abnormal adrenal progesterone production and induced estrous cycle irregularity. This is the first time tha reproductive toxicity of cyantraniliproleis demonstrated, and most of the results are due to its capacity in causing oxidative stress.

All these presented conditions could compromise reproductive capacity, and also lead to infertility. Further studies are necessary for a better understanding the mechanisms of the action of cyantraniliprole on the female genital tract of mammals.

Acknowledgment

The authors are grateful to CAPES (Coordinating Body for the Improvement of Postgraduate Studies in Higher Education) for providing a PhD scholarship to Scarton, S.R.S and partially financial support (Finance Code 001). This paper represents part of the PhD thesis by Scarton, S.R.S. (State University of Londrina - Brazil) under the supervision of GSA Fernandes.

Conflicts of interest

The authors declare no conflicts of interest.

6.0. References

[1] W.L. Soares, L.N. da Cunha, M.F. de S. Porto, Uma política de Incentivo fiscal a agrotóxicos no Brasil é injustificável e insustentável, (2020) 59. https://www.abrasco.org.br/site/wp-content/uploads/2020/02/Relatorio-Abrasco-Desoneracao-Fiscal-Agrotoxicos-17.02.2020.pdf.

[2] H. Takeshima, M. Nishi, N. Iwabe, T. Miyata, T. Hosoya, I. Masai, Y. Hotta, Isolation and characterization of a gene for a ryanodine receptor/calcium release channel in Drosophila melanogaster, FEBS Lett. 337 (1994) 81–87. https://doi.org/10.1016/0014-5793(94)80634-9.

[3] A.K. Isaacs, S. Qi, R. Sarpong, J.E. Casida, Insect ryanodine receptor: Distinct but coupled insecticide binding sites for [N -C3H3]chlorantraniliprole, flubendiamide, and [3H]ryanodine, Chem. Res. Toxicol. 25 (2012) 1571–1573. https://doi.org/10.1021/tx300326m.

[4] T.P. Selby, G.P. Lahm, T.M. Stevenson, K.A. Hughes, D. Cordova, I.B. Annan, J.D. Barry, E.A. Benner, M.J. Currie, T.F. Pahutski, Discovery of cyantraniliprole, a potent and selective anthranilic diamide ryanodine receptor activator with cross-spectrum insecticidal activity, Bioorganic Med. Chem. Lett. 23 (2013) 6341–6345. https://doi.org/10.1016/j.bmcl.2013.09.076.

[5] C. Martin, K.E. Chapman, S. Thornton, R.H. Ashley, Changes in the expression of myometrial ryanodine receptor mRNAs during human pregnancy, Biochim. Biophys. Acta - Mol. Cell Res. 1451 (1999) 343–352. https://doi.org/10.1016/S0167-4889(99)00104-4.

[6] C. Martin, J.M. Hyvelin, K.E. Chapman, R. Marthan, R.H. Ashley, J.P. Savineau, Pregnant rat myometrial cells show heterogeneous ryanodine- and caffeine-sensitive calcium stores, Am. J. Physiol. - Cell Physiol. 277 (1999)

243–252. https://doi.org/10.1152/ajpcell.1999.277.2.c243.

[7] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21. https://doi.org/10.1038/35036035.

[8] S. Dikalov, Cross talk between mitochondria and NADPH oxidases, Free Radic. Biol. Med. 51 (2011) 1289–1301. https://doi.org/10.1016/j.freeradbiomed.2011.06.033.

[9] A. V. Gordeeva, R.A. Zvyagilskaya, Y.A. Labas, Review: Cross-talk between reactive oxygen species and calcium in living cells, Biokhimiya. 68 (2003) 1318–1322.

[10] A. Agarwal, A. Aponte-Mellado, B.J. Premkumar, A. Shaman, S. Gupta, The effects of oxidative stress on female reproduction: A review, Reprod. Biol. Endocrinol. 10 (2012) 1–31. https://doi.org/10.1186/1477-7827-10-49.

[11] S.D. Ray, T.S. Lam, J.A. Rotollo, S. Phadke, C. Patel, A. Dontabhaktuni, S. Mohammad, H. Lee, S. Strika, A. Dobrogowska, C. Bruculeri, A. Chou, S. Patel, R. Patel, T. Manolas, S. Stohs, Oxidative stress is the master operator of drug and chemically-induced programmed and unprogrammed cell death: Implications of natural antioxidants in vivo, BioFactors. 21 (2004) 223–232. https://doi.org/10.1002/biof.552210144.

[12] A. Agarwal, S. Gupta, R.K. Sharma, Role of oxidative stress in female reproduction, Reprod. Biol. Endocrinol. 3 (2005) 1–21. https://doi.org/10.1186/1477-7827-3-28.

[13] M. Jozwik, S. Wolczynski, M. Jozwik, M. Szamatowicz, Oxidative stress markers in preovulatory follicular fluid in humans, Mol. Hum. Reprod. 5 (1999) 409–413. https://doi.org/10.1093/molehr/5.5.409.

[14] T. Suzuki, N. Sugino, T. Fukaya, S. Sugiyama, T. Uda, R. Takaya, A. Yajima, H. Sasano, Superoxide dismutase in normal cycling human ovaries: Immunohistochemical localization and characterization, Fertil. Steril. 72 (1999) 720–726. https://doi.org/10.1016/S0015-0282(99)00332-5.

[15] M. Vega, M.C. Johnson, H.A. Díaz, L.R. Urrutia, J.L. Troncoso, L. Devoto, Regulation of human luteal steroidogenesis in vitro by nitric oxide, Endocrine. 8 (1998) 185–191. https://doi.org/10.1385/ENDO:8:2:185.

[16] N. Sugino, S. Takiguchi, S. Kashida, A. Karube, Y. Nakamura, H. Kato, Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy, Mol. Hum. Reprod. 6 (2000) 19–25. https://doi.org/10.1093/molehr/6.1.19.

[17] Y. Takagi, T. Nikaido, T. Toki, N. Kita, M. Kanai, T. Ashida, S. Ohira, I. Konishi, Levels of oxidative stress and redox-related molecules in the placenta in preeclampsia and fetal growth restriction, Virchows Arch. 444 (2004) 49–55. https://doi.org/10.1007/s00428-003-0903-2.

[18] A.L. Tranquilli, V. Bezzeccheri, S.R. Giannubilo, C. Scagnoli, L. Mazzanti, G.G. Garzetti, Amniotic vascular endothelial growth factor (VEGF) and nitric oxide (NO) in women with subsequent preeclampsia, Eur. J. Obstet. Gynecol. Reprod. Biol. 113 (2004) 17–20. https://doi.org/10.1016/S0301-2115(03)00369-5.

[19] L. Ferraz, C.A.B. Ramos, A. Braga, L.G.C. Velarde, K.M. Elias, N.S. Horowitz, P.F. Lopes, R.S. Berkowitz, Association between antioxidant vitamins and oxidative stress among patients with a complete hydatidiform mole, Clinics. 75 (2020) 1–6. https://doi.org/10.6061/clinics/2020/e1724.

[20] R.B. Ness, C. Cottreau, Possible role of ovarian epithelial inflammation in

ovarian cancer, J. Natl. Cancer Inst. 91 (1999) 1459–1467. https://doi.org/10.1093/jnci/91.17.1459.

[21] J. Zhang, Y. Bao, X. Zhou, L. Zheng, Polycystic ovary syndrome and mitochondrial dysfunction, Reprod. Biol. Endocrinol. 17 (2019) 1–15. https://doi.org/10.1186/s12958-019-0509-4.

[22] S. Amreen, P. Kumar, P. Gupta, P. Rao, Evaluation of oxidative stress and severity of endometriosis, J. Hum. Reprod. Sci. 12 (2019) 40–46. https://doi.org/10.4103/jhrs.JHRS_27_17.

[23] C. Chen, Y. Zhou, C. Hu, Y. Wang, Z. Yan, Z. Li, R. Wu, Mitochondria and oxidative stress in ovarian endometriosis, Free Radic. Biol. Med. 136 (2019) 22–34. https://doi.org/10.1016/j.freeradbiomed.2019.03.027.

[24] S. Gupta, A. Agarwal, J. Banerjee, J.G. Alvarez, The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: A systematic review, Obstet. Gynecol. Surv. 62 (2007) 335–347. https://doi.org/10.1097/01.ogx.0000261644.89300.df.

[25] M. Abdollahi, A. Ranjbar, S. Shadnia, S. Nikfar, A. Rezaie, Pesticides and oxidative stress: A review, Med. Sci. Monit. 10 (2004) 141–148.

[26] M.P. Green, A.J. Harvey, B.J. Finger, G.A. Tarulli, Endocrine disrupting chemicals : Impacts on human fertility and fecundity during the peri-conception period, Environ. Res. 194 (2021) 110694. https://doi.org/10.1016/j.envres.2020.110694.

[27] S. Rattan, C. Zhou, C. Chiang, S. Mahalingam, E. Brehm, J.A. Flaws, Exposure to endocrine disruptors during adulthood: consequences for female fertility, J. Endocrinol. 233 (2017) R109–R129. https://doi.org/10.1530/JOE-17-0023.

[28] T.T. Schug, A. Janesick, B. Blumberg, J.J. Heindel, Endocrine disrupting chemicals and disease susceptibility, J. Steroid Biochem. Mol. Biol. 127 (2011) 204–215. https://doi.org/10.1016/j.jsbmb.2011.08.007.

[29] P. Sutton, D. Wallinga, J. Perron, M. Gottlieb, L. Sayre, T. Woodruff, Reproductive Health And The Industrialized Food System: A Point Of Intervention For Health Policy, Health Aff. 30 (2011) 888–897. https://doi.org/10.1377/hlthaff.2010.1255.

[30] Z.C. Luo, J.M. Liu, W.D. Fraser, Large prospective birth cohort studies on environmental contaminants and child health - Goals, challenges, limitations and needs, Med. Hypotheses. 74 (2010) 318–324. https://doi.org/10.1016/j.mehy.2009.08.044.

[31] F.K. MARCONDES, F.J. BIANCHI, A.P. TANNO, Determination of the estrous cycle phases of rats: some helpful considerations, Brazilian J. Biol. 62 (2002) 609–614. https://doi.org/10.1590/S1519-69842002000400008.

[32] M.T. Guerra, W.R. Scarano, F.C. de Toledo, J.A.A. Franci, W.D.G. Kempinas, Reproductive development and function of female rats exposed to di-η-butyl-phthalate (DBP) in utero and during lactation, Reprod. Toxicol. 29 (2010) 99–105. https://doi.org/10.1016/j.reprotox.2009.10.005.

[33] C. Borgeest, D. Symonds, L.P. Mayer, P.B. Hoyer, J.A. Flaws, Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse, Toxicol. Sci. 68 (2002) 473–478. https://doi.org/10.1093/toxsci/68.2.473.

[34] C.E. Talsness, M. Shakibaei, S.N. Kuriyama, S.W. Grande, A. Sterner-Kock, P. Schnitker, C. De Souza, K. Grote, I. Chahoud, Ultrastructural changes observed in rat ovaries following in utero and lactational exposure to low doses

of a polybrominated flame retardant, Toxicol. Lett. 157 (2005) 189–202. https://doi.org/10.1016/j.toxlet.2005.02.001.

[35] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

[36] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

[37] O. V. Lushchak, O.I. Kubrak, O. V. Lozinsky, J.M. Storey, K.B. Storey, V.I. Lushchak, Chromium(III) induces oxidative stress in goldfish liver and kidney, Aquat. Toxicol. 93 (2009) 45–52. https://doi.org/10.1016/j.aquatox.2009.03.007.

[38] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 71 (1975) 012004. https://doi.org/10.1088/1742-6596/71/1/012004.

[39] H. Habig, J. Pabst, B. Jakoby, Glutathione AA from Rat Liver The glutathione, RCHIVES Biochem. Biophys. 175 (1976) 710–716. https://doi.org/10.1016/0003-9861(76)90563-4.

[40] R.K. Crouch, S.E. Gandy, G. Kimsey, R.A. Galbraith, G.M.P. Galbraith, M.G. Buse, The Inhibition of Islet Superoxide Dismutase by Diabetogenic Drugs, Diabetes. 30 (1981) 235–241. https://doi.org/http://dx.doi.org/10.2337/diab.30.3.235.

[41] L. Flohé, W.A. Günzler, Assays of glutathione peroxidase, Methods Enzym. 105 (1984) 114–120. https://doi.org/10.1016/S0076-6879(84)05015-1.

[42] A. Sies, H.; R. Kock, O; Martino, E.; Boveris, Increased biliary glutathione disulfide release in chronically ethanol treated rats, Febs Lett. 103 (1979) 287–

290. https://doi.org/10.1016/0014-5793(79)81346-0.

[43] A.C. Gore, V.A. Chappell, S.E. Fenton, J.A. Flaws, A. Nadal, G.S. Prins, J. Toppari, R.T. Zoeller, EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals, Endocr. Rev. 36 (2015) 1–150. https://doi.org/10.1210/er.2015-1010.

[44] D.M. Grant, Detoxification Pathways in the Liver, J. Inherit. Metab. Dis. 14 (1991) 421–430. https://doi.org/10.1007/978-94-011-9749-6_2.

[45] H. Cichoz-Lach, A. Michalak, Oxidative stress as a crucial factor in liver diseases, World J. Gastroenterol. 20 (2014) 8082–8091. https://doi.org/10.3748/wjg.v20.i25.8082.

[46] S. Fritz, K.J. Föhr, S. Boddien, U. Berg, C. Brucker, A. Mayerhofer, Functional and molecular characterization of a muscarinic receptor type and evidence for expression of choline-acetyltransferase and vesicular acetylcholine transporter in human granulosa-luteal cells, J. Clin. Endocrinol. Metab. 84 (1999) 1744–1750. https://doi.org/10.1210/jc.84.5.1744.

[47] M.E. Cruz, A. Flores, B.E. Alvarado, C.G. Hernández, A. Zárate, R. Chavira, M. Cárdenas, I. Arrieta-Cruz, R. Gutiérrez-Juárez, Ovulation requires the activation on proestrus of M1 muscarinic receptors in the left ovary, Endocrine. 49 (2015) 809–819. https://doi.org/10.1007/s12020-014-0524-3.

[48] S.R. Guraya SS1, Kaur P, Histochemical and biochemical studies on esterase activity in the rat ovary., Eur. J. Morphol. 29 (1991) 161–172.

[49] J. Urra, J. Blohberger, M. Tiszavari, A. Mayerhofer, H.E. Lara, In vivo blockade of acetylcholinesterase increases intraovarian acetylcholine and enhances follicular development and fertility in the rat, Sci. Rep. 6 (2016) 1–9. https://doi.org/10.1038/srep30129.

[50] E.-B.M.. Mustafa F., Fatani J.A., El-Eishi H., Intrinsic Innervation of the Uterus in Guinea Pig and Rat, Acta Anat. 5 (1987) 129:53.

[51] Y. Sato, H. Hotta, H. Nakayama, H. Suzuki, Sympathetic and parasympathetic regulation of the uterine blood flow and contraction in the rat, J. Auton. Nerv. Syst. 59 (1996) 151–158. https://doi.org/10.1016/0165-1838(96)00019-7.

[52] M. HAMMARSTRÖM, N.O. SJÖSTRAND, Evidence for a cholinergic secretory innervation of the guinea–pig endometrium, Acta Physiol. Scand. 106 (1979) 11–15. https://doi.org/10.1111/j.1748-1716.1979.tb06363.x.

[53] F.M.F. Abdalla, E. Maróstica, Z.P. Picarelli, L.C. Abreu, M.C.W. Avellar, C.S. Porto, Effect of estrogen on muscarinic acetylcholine receptor expression in rat myometrium, Mol. Cell. Endocrinol. 213 (2004) 139–148. https://doi.org/10.1016/j.mce.2003.10.040.

[54] G.J.M. Den Hartog, E. Vegt, W.J.F. Van Der Vijgh, G.R.M.M. Haenen, A. Bast, Hypochlorous acid is a potent inhibitor of acetylcholinesterase, Toxicol. Appl. Pharmacol. 181 (2002) 228–232. https://doi.org/10.1006/taap.2002.9419.

[55] Y. Liu, Z. Ding, Obesity, a serious etiologic factor for male subfertility in modern society, Reproduction. 154 (2017) R123–R131. https://doi.org/10.1530/REP-17-0161.

[56] K.U. Schallreuter, S. Elwary, Hydrogen peroxide regulates the cholinergic signal in a concentration dependent manner, Life Sci. 80 (2007) 2221–2226. https://doi.org/10.1016/j.lfs.2007.01.028.

[57] E.O. Farombi, A.O. Abolaji, I.A. Adedara, I. Maduako, I. Omodanisi, Artemisinin induces hormonal imbalance and oxidative damage in the erythrocytes and uterus but not in the ovary of rats, Hum. Exp. Toxicol. 34 (2015) 83–92. https://doi.org/10.1177/0960327114532385.

[58] M. Ushio-Fukai, R.W. Alexander, Reactive oxygen species as mediators of angiogenesis signaling. Role of NAD(P)H oxidase, Mol. Cell. Biochem. 264 (2004) 85–97. https://doi.org/10.1023/B:MCBI.0000044378.09409.b5.

[59] K. V. Premkumar, S.K. Chaube, Increased level of reactive oxygen species persuades postovulatory aging-mediated spontaneous egg activation in rat eggs cultured in vitro, Vitr. Cell. Dev. Biol. - Anim. 52 (2016) 576–588. https://doi.org/10.1007/s11626-016-0007-3.

[60] K. Shkolnik, A. Tadmor, S. Ben-Dor, N. Nevo, D. Galiani, N. Dekel, Reactive oxygen species are indispensable in ovulation, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 1462–1467. https://doi.org/10.1073/pnas.1017213108.

[61] Y.C. Awasthi, D.D. Dao, R.P. Saneto, Interrelationship between anionic and cationic forms of glutathione S-transferases of human liver., Biochem. J. 191 (1980) 1–10. https://doi.org/10.1042/bj1910001.

[62] S.S. Singhal, B.F. Godley, A. Chandra, U. Pandya, G.F. Jin, M.K. Saini, S. Awasthi, Y.C. Awasthi, Induction of glutathione S-transferase hGST 5.8 is an early response to oxidative stress in RPE cells., Invest. Ophthalmol. Vis. Sci. 40 (1999) 2652–9. http://www.ncbi.nlm.nih.gov/pubmed/10509662.

[63] Z. Cao, D. Hardej, L.D. Trombetta, Y. Li, The role of chemically induced glutathione and glutathione S-transferase in protecting against 4-hydroxy-2-nonenal-mediated cytotoxicity in vascular smooth muscle cells, Cardiovasc. Toxicol. 3 (2003) 165–177. https://doi.org/10.1385/CT:3:2:165.

[64] C. Bowler, M. Van Montagu, D. Inze, Superoxide Dismutase and Stress Tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83–116. https://doi.org/10.1146/annurev.pp.43.060192.000503.

[65] J.R. Arthur, The glutathione peroxidases, Cell. Mol. Life Sci. 57 (2000) 1825–

1835. https://doi.org/10.1007/pl00000664.

[66] S. Salzano, P. Checconi, E.M. Hanschmann, C.H. Lillig, L.D. Bowler, P. Chan, D. Vaudry, M. Mengozzi, L. Coppo, S. Sacre, K.R. Atkuri, B. Sahaf, L.A. Herzenberg, L.A. Herzenberg, L. Mullen, P. Ghezzi, Linkage of inflammation and oxidative stress via release of glutathionylated peroxiredoxin-2, which acts as a danger signal, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 12157–12162. https://doi.org/10.1073/pnas.1401712111.

[67] V.O. Kaminskyy, B. Zhivotovsky, Free Radicals in Cross Talk Between Autophagy and Apoptosis, Antioxid. Redox Signal. 21 (2014) 86–102. https://doi.org/10.1089/ars.2013.5746.

[68] J.C. Patterson, B.A. Joughin, B. van de Kooij, D.C. Lim, D.A. Lauffenburger, M.B. Yaffe, ROS and Oxidative Stress Are Elevated in Mitosis during Asynchronous Cell Cycle Progression and Are Exacerbated by Mitotic Arrest, Cell Syst. 8 (2019) 163-167.e2. https://doi.org/10.1016/j.cels.2019.01.005.

[69] T.P. Szatrowski, C.F. Nathan, Production of Large Amounts of Hydrogen Peroxide by Human Tumor Cells, Cancer Res. 51 (1991) 794–798.

[70] L. Lzr, The Role of Oxidative Stress in Female Reproduction and Pregnancy, in: Oxidative Stress Dis., InTech, 2012. https://doi.org/10.5772/32515.

[71] S. Prasad, M. Tiwari, A.N. Pandey, T.G. Shrivastav, S.K. Chaube, Impact of stress on oocyte quality and reproductive outcome, J. Biomed. Sci. 23 (2016) 19–23. https://doi.org/10.1186/s12929-016-0253-4.

[72] H. Yang, Y. Xie, D. Yang, D. Ren, Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma, Oncotarget. 8 (2017) 25310–25322. https://doi.org/10.18632/oncotarget.15813.

[73] R. Prasad, L.A. Metherell, A.J. Clark, H.L. Storr, Deficiency of ALADIN impairs

redox homeostasis in human adrenal cells and inhibits steroidogenesis, Endocrinology. 154 (2013) 3209–3218. https://doi.org/10.1210/en.2013-1241.

[74] Z. Shi, Y. Feng, J. Wang, H. Zhang, L. Ding, J. Dai, Perfluorododecanoic acid-induced steroidogenic inhibition is associated with steroidogenic acute regulatory protein and reactive oxygen species in cAMP-stimulated leydig cells, Toxicol. Sci. 114 (2010) 285–294. https://doi.org/10.1093/toxsci/kfq014.

[75] M.A. Gómez-Zubeldia, S. Corrales, J. Arbués, A.G. Nogales, J.C. Millán, Influence of estradiol and gestagens on oxidative stress in the rat uterus, Gynecol. Oncol. 86 (2002) 250–258. https://doi.org/10.1006/gyno.2002.6753.

[76] V. Hernández-Rabaza, R. López-Pedrajas, I. Almansa, Progesterone, lipoic acid, and sulforaphane as promising antioxidants for retinal diseases: A review, Antioxidants. 8 (2019). https://doi.org/10.3390/antiox8030053.

5 CONSIDERAÇÕES GERAIS

O presente estudo demostrou que o inseticida cyantraniliprole exerce efeitos negativos sobre aspectos do trato genital masculino e feminino. Essa condição deve ser levada em consideração visto a ampla possibilidade de uso dessa substância. Entendemos que medidas educativas e de novas políticas de saúde pública são necessárias para o incentivo do uso adequado deste produto. É importante ressaltar que mesmo a baixa dose é suficiente para causar danos permanentes que condizem com a diminuição da fertilidade masculina e feminina.

REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO

ARAÚJO, Alberto José De et al. Exposição múltipla a agrotóxicos e efeitos à saúde: estudo transversal em amostra de 102 trabalhadores rurais, Nova Friburgo, RJ. **Ciência & Saúde Coletiva**, *[S. l.]*, v. 12, n. 1, p. 115–130, 2007. DOI: 10.1590/S1413-81232007000100015. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1413-81232007000100015&lng=pt&tlng=pt.

BENTON, Lauri; SHAN, Li Xin; HARDY, Matthew P. Differentiation of adult Leydig cells. **Journal of Steroid Biochemistry and Molecular Biology**, *[S. l.]*, v. 53, n. 1–6, p. 61–68, 1995. DOI: 10.1016/0960-0760(95)00022-R.

BLAIR, Aaron et al. **Disease and injury among participants in the Agricultural Health StudyJournal of Agricultural Safety and Health**, 2005.

BOEKELHEIDE, Kim; JOHNSON, Kamin J.; RICHBURG, John H. Sertoli Cell Toxicants. *In*: **Sertoli Cell Biology**. [s.l.] : Elsevier, 2005. p. 345–382. DOI: 10.1016/B978-012647751-1/50021-0. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/B9780128012383021371.

BOUÉ, Joëlle; BOUÉ, André; LAZAR, Philippe. Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions.

Teratology, *[S. l.]*, v. 12, n. 1, p. 11–26, 1975. DOI: 10.1002/tera.1420120103.

BRASIL. MINISTÉRIO DA SAÚDE. **Posicionamento do Instituto Nacional de Câncer José Alencar Gomes da Silva acerca dos agrotóxicosInca**, 2015. DOI: 978-85-7318-283-5. Disponível em: http://www1.inca.gov.br/inca/Arquivos/comunicacao/posicionamento_do_inca_sobre_os_agrotoxicos_06_abr_15.pdf.

BREMNER, W. J.; MILLAR, M. R.; SHARPE, R. M.; SAUNDERS, P. T. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. **Endocrinology**, *[S. l.]*, v. 135, n. 3, p. 1227–1234, 1994. DOI: 10.1210/endo.135.3.8070367. Disponível em: https://academic.oup.com/endo/article-lookup/doi/10.1210/endo.135.3.8070367.

CARREAU, Serge; WOLCZYNSKI, Slaweck; GALERAUD-DENIS, Isabelle. **Aromatase, oestrogens and human male reproduction.Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, 2010. DOI: 10.1098/rstb.2009.0113.

DACHEUX, Jean-louis; DACHEUX, Françoise. New insights into epididymal function in relation to sperm maturation. **REPRODUCTION**, *[S. l.]*, v. 147, n. 2, p. R27–R42, 2014. DOI: 10.1530/REP-13-0420. Disponível em: https://rep.bioscientifica.com/view/journals/rep/147/2/R27.xml.

DIXON, Darlene et al. **Nonproliferative and proliferative lesions of the rat and**

mouse female reproductive systemJournal of Toxicologic Pathology, 2014. DOI: 10.1293/tox.27.1S.

DIXON, Darlene; VIDAL, Justin D.; LEININGER, Joel R.; JOKINEN, Micheal P. **Oviduct, Uterus, and Vagina**. [s.l.] : Elsevier Inc., 2018. DOI: 10.1016/b978-0-12-391448-4.00027-7. Disponível em: http://dx.doi.org/10.1016/B978-0-12-391448-4.00027-7.

DUPONT DO BRASIL S/A. **Ficha de informações de segurança de produtos químicos - Benevia®**, 2016. Disponível em: http://downloads.labsynth.com.br/FISPQ/rv2012/FISPQ- Anilina.pdf.

FOLEY, GEORGE L. Overview of male reproductive pathology. **Toxicologic pathology**, *[S. l.]*, v. 29, n. 1, p. 49–63, 2001.

FRANÇA, L. R.; HESS, R. A.; DUFOUR, J. M.; HOFMANN, M. C.; GRISWOLD, M. D. The Sertoli cell: one hundred fifty years of beauty and plasticity. **Andrology**, *[S. l.]*, v. 4, n. 2, p. 189–212, 2016. DOI: 10.1111/andr.12165. Disponível em: http://doi.wiley.com/10.1111/andr.12165.

FRANÇA, Luiz R.; AVELAR, Gleide F.; ALMEIDA, Fernanda F. L. **Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigsTheriogenology**, 2005. DOI: 10.1016/j.theriogenology.2004.09.014.

GRISWOLD, M. D. **The central role of Sertoli cells in spermatogenesis.Seminars in cell & developmental biology**, 1998. DOI: 10.1006/scdb.1998.0203.

KAKAR, Sham S.; MALIK, M. Tariq; MAZHAWIDZA, Williard. **Gonadotropin-Releasing Hormone Receptors: Structure, Expression, and Signaling TransductionVitamins and Hormones**, 2004.

KERR, J. B.; LOVELAND, K. L.; O'BRYAN, M. K.; DE KRETSER, D. M. **Cytology of the testis and intrinsic control mechanismsKnobil and Neill's Physiology of Reproduction**, 2006. DOI: 10.1016/B978-012515400-0/50023-3.

KORENBROT, C. C.; HUHTANIEMI, I. T.; WEINER, R. I. Preputial Separation as an External Sign of Pubertal Development in the Male Rat1. **Biology of Reproduction**, *[S. l.]*, v. 17, n. 2, p. 298–303, 1977. DOI: 10.1095/biolreprod17.2.298. Disponível em: https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod17.2.298.

KRAPF, Dario et al. **CSrc is necessary for epididymal development and is incorporated into sperm during epididymal transitDevelopmental Biology**Elsevier, , 2012. DOI: 10.1016/j.ydbio.2012.06.017. Disponível em: http://dx.doi.org/10.1016/j.ydbio.2012.06.017.

LEBLOND, C. P.; CLERMONT, Y. **Definition of the stages of the cycle of the seminiferous epithelium in the rat.Annals of the New York Academy of Sciences**, 1952. DOI: 10.1111/j.1749-6632.1952.tb26576.x.

LEVINE, Jon E. Neuroendocrine Control of the Ovarian Cycle of the Rat. *In*: **Knobil and Neill's Physiology of Reproduction**. Fourth Edi ed. [s.l.] : Elsevier, 2015. p. 1199–1257. DOI: 10.1016/B978-0-12-397175-3.00026-0. Disponível em: http://dx.doi.org/10.1016/B978-0-12-397175-3.00026-0%5Cnhttp://linkinghub.elsevier.com/retrieve/pii/B9780123971753000260.

LONDRES, Flávia. **Agrotóxicos no Brasil - Um guia para a ação em defesa da vida**Rio de JaneiroArticulação Nacional de Agroecologia, , 2011. Disponível em: http://aspta.org.br/wp-content/uploads/2011/09/Agrotoxicos-no-Brasil-mobile.pdf.

MRUK, Dolores D.; CHENG, C. Yan. Sertoli-sertoli and sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. **Endocrine Reviews**, *[S. l.]*, v. 25, n. 5, p. 747–806, 2004. DOI: 10.1210/er.2003-0022.

OJEDA, S. R.; ANDREWS, W. W.; ADVIS, J. P.; WHITE, S. Smitw. Recent advances in the endocrinology of puberty. **Endocrine Reviews**, *[S. l.]*, v. 1, n. 3, p. 228–257, 1980. DOI: 10.1210/edrv-1-3-228.

OJEDA, Sergio R.; SKINNER, Michael K. **Puberty in the ratKnobil and Neill's Physiology of Reproduction**, 2006. DOI: 10.1016/B978-012515400-0/50043-9.

PINTO, Ciro Pedro Guidotti. DIAMIDES: MODE OF ACTION AND INSECT

RESISTANCE. *In*: RIBEIRO, Julio Césas (org.). **Impacto, Excelência e Produtividade das Ciências Agrárias no Brasil 3**. 3. ed. Ponta Grossa: Atena Editora, 2020. p. 83–88. DOI: 10.22533/at.ed.4902021058. Disponível em: http://library1.nida.ac.th/termpaper6/sd/2554/19755.pdf.

PODESTÁ, Ernesto J.; RIVAROLA, Marco A. Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development. **Endocrinology**, *[S. l.]*, v. 95, n. 2, p. 455–461, 1974. DOI: 10.1210/endo-95-2-455.

ROBAIRE, B.; HINTON, B.; ORGEBINCRIST, M. The Epididymis. *In*: **Knobil and Neill's Physiology of Reproduction**. [s.l.] : Elsevier, 2006. p. 1071–1148. DOI: 10.1016/B978-012515400-0/50027-0. Disponível em: http://www.sciencedirect.com/science/article/pii/B9780125154000500270.

ROBB, G. W.; AMANN, R. P.; KILLIAN, G. J. **Daily sperm production and epididymal sperm reserves of pubertal and adult rats.Journal of reproduction and fertility**, 1978. DOI: 10.1530/jrf.0.0540103.

SHARPE, Richard M. Regulation of Spermatogenesis. *In*: **Knobil and Neill's Physiology of Reproduction**. 2° ed. New York: Raven Press, 1994. p. 1363–1434.

SILVA, Anderson Gonçalves Da; GRIGOLLI, José Fernanda Jurca. **NOVAS MOLÉCULAS SÃO ARMA PODEROSA CONTRAGEMIP**, 2016. Disponível em: www.gemip.com.br/index.php/noticias-relacionadas/55-novas-moleculas-sao-arma-poderosa-contra-lagartas-da-soja-milho-e-algodão. Acesso em: 12 out. 2016.

SKINNER, M. K. Seminiferous Cord Formation and Germ-Cell Programming: Epigenetic Transgenerational Actions of Endocrine Disruptors. **Annals of the New York Academy of Sciences**, *[S. l.]*, v. 1061, n. 1, p. 18–32, 2005. DOI: 10.1196/annals.1336.004. Disponível em: http://doi.wiley.com/10.1196/annals.1336.004.

SMITH, Lee B.; WALKER, William H. **Hormone Signaling in the Testis**. Fourth Edi ed. [s.l.] : Elsevier, 2015. DOI: 10.1016/B978-0-12-397175-3.00016-8. Disponível em: http://dx.doi.org/10.1016/B978-0-12-397175-3.00016-8.

TAKESHIMA, Hiroshi; NISHI, Miyuki; IWABE, Naoyuki; MIYATA, Takashi; HOSOYA, Toshihiko; MASAI, Ichiro; HOTTA, Yoshiki. Isolation and characterization of a gene for a ryanodine receptor/calcium release channel in Drosophila melanogaster. **FEBS Letters**, *[S. l.]*, v. 337, n. 1, p. 81–87, 1994. DOI: 10.1016/0014-5793(94)80634-9.

TILLY, Jonathan L. Apoptosis and the ovary: A fashionable trend or food for thought? **Fertility and Sterility**, *[S. l.]*, v. 67, n. 2, p. 226–228, 1997. DOI: 10.1016/S0015-0282(97)81901-2. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0015028297819012.

WESTWOOD, F. Russell. The Female Rat Reproductive Cycle: A Practical Histological Guide to Staging. **Toxicologic Pathology**, *[S. l.]*, v. 36, n. 3, p. 375–384, 2008. DOI: 10.1177/0192623308315665.

XIAO, Xiang; MRUK, Dolores D.; WONG, Chris K. C.; YAN CHENG, C. Germ cell transport across the seminiferous epithelium during spermatogenesis. **Physiology**, *[S. l.]*, v. 29, n. 4, p. 286–298, 2014. DOI: 10.1152/physiol.00001.2014.

ZUBKOVA, Ekaterina V; ROBAIRE, Bernard. **Effect of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver and on spermatozoa motility in the Aging Brown Norway ratBiology of Reproduction**, 2004. DOI: 10.1095/biolreprod.104.028373. Disponível em: http://www.biolreprod.org/content/71/3/1002.abstract.

ANEXO A

Parecer de aprovação do projeto de pesquisa CEUA-UEL

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 134/2017 **Londrina, 30 de Novembro de 2017.**

Prezado (a) professor (a)

Certificamos que o projeto intitulado: **"Estudo dos efeitos do inseticida cyantraniliprole sobre perfil reprodutor feminino e masculino, muscular, nervoso, cardíaco, renal e hepático de ratos wistar."** protocolo CEUA **nº21106.2017.24** sob a responsabilidade de **Glaura Scantamburlo Alves Fernandes** , que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **28/11/2017**

O objetivo do projeto é analisar o efeito do cyantroniliprole sobre a morfologia do teticulo e do epidídimo, viabilidade dos espermatozóides, perfil inflamatório, função hepática, concenttração de hormônios, cérebros, corações, rins, músculos (extensor longo dos dedos, gastrocnêmio e sóleo), e sobre a regularidade do ciclo estral e morfologia de útero e ovários de ratos wistar maschos ou fêmeas. Grau de invasividade=**1**

Vigência do Projeto	06/11/2017 a 06/03/2020
Espécie/linhagem	Rato heterogênico / Wistar
Nº de animais	90 machos e 24 fêmeas.
Peso/Idade	.... / 21 dias (machos) - .... / 60 dias (fêmeas).
Sexo	Machos e fêmeas.
Origem	Biotério Central do CCB.
Amostras a serem coletadas	Sangue, testículos e epidídimos, ductos deferentes, cérebro, coração, fígado (machos e fêmeas), rins (machos e fêmeas), músculos: extensor longo dos dedos, gastrocnêmio e sóleo, útero, ovários e hipófise.

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

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

Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr (a). Glaura Scantamburlo Alves Fernandes
Responsável pelo projeto
Departamento de Biologia Geral

C/C Biotério Central do CCB
C/C para a Chefia do Depto. de Biologia Geral
C/C para a Direção do CCB

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

1
2 **ANEXO B**
3 Parecer de aprovação adendo ao projeto de pesquisa CEUA - UEL

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 93/2018 **Londrina, 07 de Junho de 2018.**

Prezado (a) professor (a)

Em resposta ao pedido de adendo referente ao processo nº 21106.2017.24 que corresponde ao projeto intitulado "**Estudo dos efeitos do inseticida cyantraniliprole sobre o perfil reprodutor feminino e masculino, nervoso, cardíaco, renal e hepático de ratos Wistar.**" sob-responsabilidade de **Glaura Scantamburlo Alves Fernandes,** o mesmo teve parecer aprovado em **04/06/2018**

Os animais previstos serão agora oriundos do Biotério Central da Universidade Estadual do Oeste do Paraná-UNIOESTE, e não mais da Universidade Estadual de Londrina. Haverá também a extração do baço dos animais para complementação da avaliação de toxicidade sistêmica.

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

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

Maria Fernanda R. Graciano
Profa. Dra. Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr. (a). Glaura Scantamburlo Alves Fernandes
Responsável pelo projeto ICCB

4