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A EXPOSIÇÃO PERIPUBERAL AO HERBICIDA À BASE DE GLIFOSATO INFLUENCIA O EFEITO DA DIETA HIPERLIPÍDICA NO MÚSCULO DIAFRAGMA DE CAMUNDONGOS ADULTOS

CASCAVEL-PR
(Dezembro/2021)

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Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Saúde – Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Biociências e Saúde.

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ANA PAULA MARCA

EFEITOS DA EXPOSIÇÃO PERIPUBERAL AO HERBICIDA À BASE DE GLIFOSATO E DA OBESIDADE INDUZIDA PELA DIETA HIPERLIPÍDICA NO MÚSCULO DIAFRAGMA DE CAMUNDONGOS ADULTOS

Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Saúde em cumprimento parcial aos requisitos para obtenção do título de Mestra em Biociências e Saúde, área de concentração Biologia, processo saúde-doença e políticas de saúde, linha de pesquisa Fatores que influenciam a morfofisiologia orgânica, APROVADO(A) pela seguinte banca examinadora:

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Ao meu filho, Bernardo Marca Nass Scalco,
você é a minha força diária.

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RESUMO

MARCA, A. P. **A exposição peripuberal ao herbicida à base de glifosato influencia o efeito da dieta hiperlipídica no músculo diafragma de camundongos adultos.** Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus Cascavel, Unioeste, 2021.

As origens desenvolvimentistas da saúde e doença estudam os efeitos causados por insultos em períodos críticos de desenvolvimento (fetal, neonatal e a puberdade), que podem comprometer a saúde ao longo da vida. O glifosato é um herbicida da classe dos organofosforados, com capacidade de disrupção endócrina e efeitos modulatórios sobre a puberdade. A exposição aos agrotóxicos em períodos críticos do desenvolvimento está relacionada com a obesidade na idade adulta. A obesidade é caracterizada como elevado índice de gordura corporal, que compromete a função de diversos sistemas. Assim, o objetivo desse estudo foi analisar os efeitos da exposição peripuberal ao herbicida à base de glifosato (GBH) sobre a histomorfometria das fibras musculares e junções neuromusculures (JNMs) do músculo diafragma de camundongos adultos obesos ou não obesos. Foram utilizados 22 camundongos machos, da linhagem C57BL/6, separados em grupos conforme à exposição ao GBH e à dieta hiperlipídica. A exposição ao GBH foi no período dos 30 aos 60 dias por gavagem, na dosagem de 50 mg/Kg/dia de Roundup®. A dieta hiperlipídica foi ofertada dos 60 aos 150 dias, assim, formaram-se 4 grupos: grupo controle (CTL, n=6); grupo herbicida à base de glifosato (GBH, n=6); grupo dieta hiperlipídica controle (DH-CTL, n=5); e grupo dieta hiperlipídica e herbicida à base de glifosato (DH-GBH, n=5). Aos 150 dias, os animais foram eutanasiados e pesados, as gorduras retroperitoneal e perigonadal e o diafragma foram coletados e pesados. Os fragmentos do músculo foram armazenados em fixadores específicos para o estudo histológico das fibras musculares e das JNMs. Na comparação entre os grupos GBH e CTL, houve redução no peso corporal dos animais GBH. Foram observadas fibras arredondadas, hipereosinofílicas e núcleos aumentados nos dois grupos, mas predominantemente no GBH. No grupo GBH, teve aumento na área, diâmetro maior e menor das fibras musculares e na área e diâmetro maior das JNMs, aumento do colágeno tipo III e diminuição da sobreposição do colágeno tipo I e III. Com relação aos grupos DH-CTL e DH-GBH, foram evidencializadas fibras arredondadas, hipereosinofílicas e vacuolização do citoplasma nos dois grupos, contudo, mais acentuadas no DH-GBH. Fibras com divisão longitudinal e hipertróficas foram visualizadas apenas no DH-GBH. Houve aumento na área e diâmetro maior das JNMs do grupo DH-GBH. Conclui-se que, a exposição peripuberal ao GBH resultou em alterações morfológicas características de degeneração muscular e alterou a morfometria das fibras musculares de camundongos adultos não obesos, alterou a morfometria das JNMs do diafragma de camundongos adultos obesos e não obesos e exacerbou as alterações degenerativas do músculo diafragma de camundongos adultos obesos.

Palavras-chave: Glifosato. Puberdade. Obesidade. Fibras Musculares Esqueléticas.

ABSTRACT

MARCA, A. P. **Peripubertal exposure to glyphosate-based herbicide influences the effect of high-fat diet on the diaphragm muscle of adult mice.** Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus Cascavel, Unioeste, 2021.

The Developmental Origins of Health and Disease studies the effects caused by insults at critical periods of development (fetal, neonatal and puberty), which can compromise health throughout life. Glyphosate is an organophosphate herbicide, with endocrine-disrupting and modulatory effects on puberty. Exposure to pesticides in critical periods of development is related to obesity in adulthood. Obesity is characterized as a high level of body fat, which compromises the function of several systems. Thus, the aim of this study was to analyze the effects of peripubertal exposure to glyphosate-based herbicide (GBH) on the histomorphometry of muscle fibers and neuromuscular junctions (NMJs) of the diaphragm muscle of obese or non-obese adult mice. Twenty-two male mice from C57BL/6 strain were used and separated into groups according to the exposure to GBH and high-fat diet. Exposure to GBH occurred from 30 to 60 days by gavage, at a dosage of 50 mg/kg/day of Roundup™. The high-fat diet was offered from 60 to 150 days, therefore, 4 groups were formed: Control Group (CTL, n=6); Glyphosate-based Herbicide Group (GBH, n=6); Control High-Fat Diet Group (DH-CTL, n=5); and High-Fat Diet And Glyphosate-based Herbicide Group (DH-GBH, n=5). At 150 days, the animals were euthanized and weighed, both retroperitoneal and perigonadal fats and the diaphragm were collected and weighed. Muscle fragments were stored in specific fixatives for the histological study of muscle fibers and NMJs. Comparing the GBH and the CTL groups, there was a reduction in the body weight of the GBH animals. Rounded, hypereosinophilic fibers and enlarged nuclei were observed in both groups, but predominantly in the GBH. In the GBH group, there was an increase in the area, larger and smaller diameter of muscle fibers and in the area and larger diameter of NMJs, an increase in type III collagen and a decrease in the overlap of type I and III collagen. Regarding the DH-CTL and DH-GBH groups, rounded, hypereosinophilic fibers and cytoplasm vacuolization were evident in both groups, however, more pronounced in the DH-GBH. Both longitudinally split and hypertrophic fibers were visualized only in the DH-GBH. There was an increase in the area and larger diameter of NMJs in the DH-GBH group. It is concluded that peripubertal exposure to GBH resulted in morphological changes that are characteristic of muscular degeneration, and altered the morphometry of muscle fibers in non-obese adult mice, altered the morphometry of diaphragm NMJs in obese and non-obese adult mice and exacerbated the degenerative changes in the diaphragm muscle of obese adult mice.

Keywords: Glyphosate. Puberty. Obesity. Skeletal Muscle Fibers.

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LISTA DE ABREVIATURAS

ACh- Acetilcolina

AChE- Acetilcolinesterase

Ca^{2+}- Cálcio

CTL- Controle

DE- Disruptor Endócrino

DEs- Disruptores Endócrinos

DH-CTL- Dieta Hiperlipídica Controle

DH-GBH- Dieta Hiperlipídica e Herbicida à Base de Glifosato

DNA - Ácido desoxirribonucleico

DOHaD- Origens Desenvolvimentistas da Saúde e Doença

DP- Dieta Padrão

EPSPs- 5-enolpiruvil-chiquimato-3-fosfato-sintase

GBH- Herbicida à Base de Glifosato

HE- Hematoxilina-Eosina

IA- Ingrediente Ativo

IBAMA- Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

JNM- Junção Neuromuscular

JNMs- Junções Neuromusculares

OF- Organofosforado

OFs- Organofosforados

SINITOX- Sistema Nacional de Informações Tóxico-Farmacológicas

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1. INTRODUÇÃO

Embora o material genético contribua para a hereditariedade e risco de doenças ao longo da vida, fatores como estilo de vida, dieta, exposição a substâncias tóxicas e experiências estressantes também podem influenciar a condição de saúde de um indivíduo, sobretudo, quando ocorrem em alta intensidade ou frequência em momentos críticos do desenvolvimento (SIDDEEK et al., 2018). Estímulos ambientais durante fases críticas do desenvolvimento podem gerar padrões fisiológicos e estruturais a longo prazo que acometem a saúde durante a vida adulta (CVITIC et al., 2018; TAPIA-MARTÍNEZ et al., 2019). A linha de pesquisa “origens desenvolvimentistas da saúde e doença” (DOHaD) visa elucidar como certas exposições precoces podem contribuir no desenvolvimento de doenças em uma fase posterior da vida (SIDDEEK et al., 2018).

Nas fases iniciais do desenvolvimento há intensa mudança nas taxas de proliferação e diferenciação celular, que determinam alterações de crescimento e morfologia. A plasticidade relacionada ao desenvolvimento permite ao organismo moldar-se em virtude de fatores ambientais, especialmente durante períodos iniciais da vida, enquanto ocorrem diferenciações celulares e o desenvolvimento de tecidos. Essa capacidade é baseada em vias moleculares que controlam a expressão gênica e a indução de fenótipos específicos na ausência de modificações na sequência do DNA (BAROUKI et al., 2012), promovendo uma programação biológica a longo prazo (GLUCKMAN et al., 2008).

Os períodos pré-natal e neonatal são as janelas críticas do desenvolvimento mais estudadas, todavia, estudos mais recentes têm indicado que outras fases da vida também são suscetíveis à programação. A puberdade é considerada um período de alta plasticidade cerebral, caracterizado por um importante crescimento e desenvolvimento, tanto físico como emocional. Experiências estressoras (agudas ou crônicas) durante esse período da vida podem resultar em alterações irreversíveis nas funções fisiológicas do organismo (PERVANIDOU; CHROUSOS, 2012).

Os disruptores endócrinos (DEs) são substâncias exógenas, que agem mesmo em concentrações extremamente baixas, alterando o funcionamento do sistema endócrino e causando efeitos deletérios, imediatos ou a longo prazo, na saúde de um organismo e/ou prole (FERLONI et al., 2019). A alteração do eixo hipotalâmico-hipofisário ou a desregulação de outros sistemas orgânicos provocada pelos pesticidas caracterizam-os como DEs (ESPINOZA-NAVARRO; PONCE-LAROSA; BUSTOS-OBREGÓN, 2017).

O uso dos pesticidas na agricultura aumentou ao longo dos anos, agravando sua toxicidade e risco de intoxicação ocupacional (SILVÉRIO et al., 2017), tornando-o um problema de saúde global (OSUNA-FLORES et al., 2019). A demanda nacional por essas substâncias cresceu 190% nos últimos dez anos, enquanto o mercado mundial cresceu 93%. Em 2008, o Brasil tornou-se o principal consumidor de agrotóxicos do mundo (CAMPOS et al., 2016).

Os pesticidas organofosforados (OFs) são os pesticidas mais usados no mundo e amplamente utilizados no Brasil (VALE et al., 2017). Sua toxicidade humana e animal compõe uma preocupação no âmbito da saúde e meio ambiente (HSU; JAN; LIANG, 2019).

A enzima neuromuscular acetilcolinesterase (AChE) é responsável pela hidrólise do neurotransmissor acetilcolina (ACh). O principal mecanismo de ação dos OFs é inibir a AChE, portanto, a presença contínua da ACh nas terminações nervosas pode promover a desregulação do sistema nervoso (HSU; JAN; LIANG, 2019; VALE et al., 2017).

O glifosato ou N- (fosfonometil) glicina, é um herbicida organofosforado (OF), de ação sistêmica, não seletivo e de amplo espectro (PANDEY; DHABADE; KUMARASAMY, 2019; REYNOSO et al., 2019). Esse composto é o principal princípio ativo contido no Roundup® (OWAGBORIAYE et al., 2019). É um agrotóxico comumente usado, sendo pulverizado em milhões de hectares em todo mundo (PANDEY; DHABADE; KUMARASAMY, 2019) e amplamente vendido no mundo (WILKENS; VALGAS; OLIVEIRA, 2019). Entretando, sua utilização exacerbada na agricultura tem contaminado o meio ambiente, deixando resíduos de glifosato no solo, água e alimentos (GILL et al., 2018; PANDEY; DHABADE; KUMARASAMY, 2019).

Essa contaminação causa preocupação, já que o glifosato possui efeitos sobre vários sistemas como endócrino, reprodutivo, urinário e neuronal (PANDEY; DHABADE; KUMARASAMY, 2019).

Dessa forma, é fundamental a compreensão e percepção individual e coletiva dos perigos apresentados pela exposição aos agrotóxicos, assim como, “[...] uma abordagem intersetorial e interdisciplinar, envolvendo saúde, agricultura, ciência e tecnologia, meio ambiente, trabalho e extensão rural [...]” (SIQUEIRA; KRUSE, 2008, p. 588) acerca do tema em questão.

O glifosato é considerado um disruptor endócrino (DE). Os DEs afetam principalmente os fetos, bebês, e as crianças em desenvolvimento, mas, na maioria das vezes, os efeitos após a exposição ao glifosato manifestam-se apenas em estágios posteriores ao desenvolvimento e/ou na fase adulta (MYERS et al., 2016).

A difícil avaliação dos efeitos da exposição prolongada a agrotóxicos em baixas doses é justificada pela ausência de manifestações clínicas. Contudo, a exposição crônica a pesticidas apresenta riscos elevados para os indivíduos (SILVÉRIO et al., 2017). Estudos apontam efeitos adversos do glifosato a longo prazo no organismo, como lesões no fígado e rins (MESNAGE et al., 2015; ZHU et al., 2012), efeito carcinogênico (GUYTON et al., 2015), surgimento de parkisionismo (BARBOSA, E. R. et al., 2001; ERIGUCHI et al., 2019), surgimento de neoplasias (SCHIMPF et al., 2017) e alterações na espermatogênese e nos níveis de testosterona (DALLEGRAVE et al., 2003).

As principais vias de exposição humana às substâncias tóxicas são a via gastrintestinal por ingestão, a respiratória através da inalação e a cutânea. Sendo as vias pulmonares e cutâneas as mais relevantes na intoxicação ocupacional e ambiental. Através da inibição da AChE, os inseticidas OFs podem ocasionar constrição brônquica e aumento de secreções, e, consequentemente, podem resultar em insuficiência respiratória (MAGALHÃES, 2017). Em geral, as alterações decorrentes da ação dos OFs nos músculos estriados esqueléticos dependem da estrutura química do composto e do tipo de fibra muscular, contudo, o diafragma e os músculos intercostais são os principais acometidos (SIQUEIRA; KRUSE, 2008).

O diafragma é o principal músculo inspiratório (BARBOSA et al., 2019), do qual o comando é simultaneamente automático e voluntário (SOUCHARD, 1989). O

comprometimento da musculatura esquelética, principalmente musculatura respiratória, causado pela intoxicação por OF, possivelmente contribui para o alto índice de morbidade nessas intoxicações (CAVALIERE et al., 1996).

A obesidade é uma doença caracterizada por um elevado índice de gordura corporal (BOUDALIA et al., 2021; NEWBOLD et al., 2008), que pode resultar em diversos efeitos negativos sobre o organismo de um indivíduo (NEWBOLD et al., 2008), inclusive, sobre o sistema músculo esquelético (PETROVICS et al., 2021; YAN et al., 2021).

Assim, levanta-se a hipótese de que a exposição peripuberal ao GBH possa promover o dessaranjo do padrão fascicular das fibras musculares e, ainda, aumentar o tamanho da área das junções neuromusculares (JNMs) do diafragma de camundongos adultos, obesos ou não.

2. OBJETIVOS

2.1 Objetivo Geral

Analisar os efeitos da exposição peripuberal ao herbicida à base de glifosato sobre a histomorfometria das fibras musculares e junções neuromusculures (JNMs) do músculo diafragma de camundongos adultos, obesos ou não obesos.

2.2 Objetivos Específicos

Estudar as características do músculo diafragma de camundongos adultos, obesos ou não, expostos à administração do GBH durante o período peripuberal, quanto à:

- Estrutura morfológica das fibras musculares;
- Área, diâmetro maior e menor das fibras musculares;
- Alterações no tecido conjuntivo;
- Área, diâmetros maior e menor das JNMs.

3. REVISÃO DE LITERATURA

3.1 Origens desenvolvimentistas da saúde e doença (DOHaD)

O termo programação metabólica refere-se aos estímulos ou insultos recebidos em uma fase crítica da vida, compreendida desde a vida fetal até a adolescência ou puberdade. Esses períodos sensíveis são caracterizados por uma alta plasticidade, podendo resultar em alterações estruturais e funcionais permanentes em um organismo (SILVA et al., 2015). Portanto, a exposição a condições adversas em fases críticas do desenvolvimento podem acarretar em prejuízos futuros irreversíveis para a saúde, sugerindo uma programação biológica a longo prazo (GLUCKMAN et al., 2008).

David Baker, em 1986, descreveu o termo “programação” fundamentado na plasticidade relacionada ao desenvolvimento em que os agravos ambientais ou ocorridos na vida fetal pudessem induzir a um mecanismo epigenético, no qual um único genótipo origina diferentes fenótipos. Sendo assim, tanto o ambiente celular quanto as relações do ser com o ambiente podem acometer questões comportamentais, morfológicas e a expressão gênica, sendo capaz de ocorrer em questão de horas (GANU et al., 2012; SILVEIRA et al., 2007).

Sendo assim, em casos de insultos, visando a sobrevivência, o feto teria capacidade adaptativa intrautero, através da otimização do consumo de substâncias essênciais reduzidas. Contudo, essa adaptação beneficiaria metabolicamente órgãos nobres em prejuízo de outros, modificando o desenvolvimento e função dos tecidos de maneira prolongada (SILVEIRA et al., 2007). Além disso, a programação permite que o organismo em fase de desenvolvimento adote um fenótipo que se adapte melhor ao ambiente. No entanto, os desiquilíbrios dessas adaptações podem resultar em maior suscetibilidade ao desenvolvimento de patologias ao longo da vida (PADMANABHAN; CARDOSO; PUTTABYATAPPA, 2016).

Tendo como base estudos no ramo da programação, inclusive os estudos de Barker supracitados, foram estabelecidos novos conceitos que exploram a relação entre os insultos sofridos em fases críticas da vida e a condição de saúde no futuro, sendo atualmente denominada DOHaD (LAKER et al., 2013; SILVEIRA et al., 2007; SUZUKI, 2017).

A DoHaD é uma área da ciência que aprofunda-se em elucidar os efeitos causados por eventos adversos (insultos) não apenas em período pré natal, mas sim, em todo o período de desenvolvimento, que podem resultar em mudanças fisiológicas e, consequentemente, afetar o padrão de saúde e doença por toda sua vida (GLUCKMAN; HANSON; MITCHELL, 2010; MARQUES et al., 2021; SUZUKI, 2017).

Os estudos do DoHaD que originalmente começaram com a nutrição no período pré natal evoluíram e incluíram diversos fatores ambientais internos e externos que afetam o corpo. Dentre eles: fatores ambientais físicos, químicos e biológicos, como drogas, medicamentos, poluentes, produtos químicos, luz, sons, microbiota intestinal, estresse mental e físico, entre outros (SUZUKI, 2017).

O paradigma DOHaD associado a epigenética (tendo como base o dogma central da biologia molecular), tornou-se um conceito central nas áreas de médicina e ciências biológicas (SUZUKI, 2017).

O campo da epigenética tem potencial para explicar as hipóteses das DoHaD (SILVEIRA et al., 2007; SUZUKI, 2017). As mudanças epigenéticas são moduladas a partir da exposição ambiental, ou seja, a epigenética é considerada a interface entre a genética e o meio em que se vive (MARQUES et al., 2021).

Alterações na metilação do DNA, mudanças estruturais da cromatina através de acetilação de histonas e na expressão de microRNAs são exemplos de mecanismos epigenéticos envolvidos na programação metabólica (BAROUKI et al., 2012; LANE, 2014), sendo que, as alterações epigenéticas podem, inclusive, ultrapassar gerações (SILVEIRA et al., 2007).

A programação pode ocorrer em diferentes estágios iniciais da vida, também denominados períodos críticos de desenvolvimento, sendo eles: vida pré-natal, pós-natal, infância, puberdade ou adolescência. Esses períodos são marcados por um

alto grau de plasticidade (CERF; LOUW; HERRERA, 2015; HO et al., 2016; SILVEIRA et al., 2007).

A puberdade é um período de crescimento infantil que precede o início da adolescência (DUTTA; SENGUPTA, 2016), é um processo de mudanças pelas quais os indivíduos atingem a maturidade sexual e tornam-se capazes de reproduzir (LUCACCIONI et al., 2020), compreende uma fase de evolução física, desenvolvimento emocional e grande plasticidade cerebral (DUTTA; SENGUPTA, 2016).

Com relação à idade, pode ser considerado normal o início puberal entre 8 e 13 anos nas meninas, e entre 9,5 anos e 13,5 anos nos meninos (CARGNELUTTI et al., 2021; LUCACCIONI et al., 2020). Nessa fase, ocorrem mudanças estruturais e funcionais no hipotálamo e no eixo hipotálamo-hipófise-adrenal decorrentes das novas conexões neuronais e aumento dos hormônios sexuais. Essas mudanças podem interferir na modulação do controle do peso e metabolismo na fase adulta (BLAKEMORE; BURNETT; DAHL, 2010; PERVANIDOU; CHROUSOS, 2012).

Pervanidou e Chrousos (2012), reiteram que experiências estressoras durante essa fase crítica da vida podem ter efeitos frequentemente irreversíveis em longo prazo na emoção, comportamento, crescimento, metabolismo, função reprodutiva, imunológica e cardiovascular.

A puberdade nos ratos ocorre dos 30 aos 40 dias, enquanto a maturação sexual entorno dos 50 aos 60 dias (ANDRADE; PINTO; OLIVEIRA, 2006). Nos machos, ela é considerada um fenômeno continuado e é confirmada através da avaliação de separação balanoprepucial, quando é constatada a separação da mucosa prepucial e exposição da glande peniana (OJEDA; SKINNER, 2006; PARKER; SCHIMMER, 2006; ROMANO et al., 2008).

Condições de estresse crônicos durante a infância e a adolescência podem levar à depressão e à ansiedade, bem como à obesidade e à síndrome metabólica, o que pode estar relacionado à uma disfunção do eixo hipotálamo-hipófise-adrenal (RIBEIRO et al., 2019). Além disso, estudos demonstraram alterações permanentes na função cerebral de ratos submetidos a estímulos estressantes no período puberal (TSOORY; GUTERMAN; RICHTER-LEVIN, 2010).

Contudo, são escassos os estudos desenvolvidos utilizando diferentes insultos nesse período específico do desenvolvimento. Mao et al., (2018) evidenciaram que a exposição de ratas ao herbicida glifosato durante a gestação, em doses consideradas seguras, é capaz de modificar a microbiota intestinal da prole durante o início do desenvolvimento, principalmente na fase peripuberal. Esses achados justificam futuros estudos sobre os potenciais efeitos do glifosato na saúde durante o início do desenvolvimento.

Outro estudo demonstrou que a prole de camundongos expostos diariamente ao composto OF à base de *diazinon* durante a gestação resultou em alterações de comportamento e desenvolvimento, prejuízos na resistência, coordenação e função motora, sugerindo assim, disfunção neuromuscular e neurocomportamental (SPYKER; AVERY, 1977).

3.2 Disruptores Endócrinos (DEs)

DE é uma substância ou mistura exógena que altera as funções do sistema endócrino e, consequentemente, causa efeitos adversos à saúde de um organismo ou da sua prole (WHO/UNEP, 2013).

Os hormônios e suas vias de sinalização são essenciais para o funcionamento de todos os tecidos e órgãos. O sistema endócrino é responsavel por controlar diversos processos no corpo, incluindo o desenvolvimento, reprodução, metabolismo, imunidade e comportamento, esses processos dependem da perfeita ação dos hormônios no organismo. Os DEs interferem no sistema endócrino e na atividade hormonal normal e os seus efeitos nocivos na saúde podem ser notados muito tempo após o fim da exposição, inclusive, podem gerar consequências para as gerações futuras (MONNERET, 2017).

Os DEs representam um risco à saúde, atuando por meio de diferentes mecanismos de ação (LUCACCIONI et al., 2020), podendo ser por meio da substituição dos hormônios naturais, bloqueio da ação hormonal ou aumento ou diminuição dos níveis hormonais normais (SANTAMARTA, 2001). E os mecanismos de ação pelos quais transmitem efeitos deletérios às gerações futuras incluem as modificações epigenéticas (INGARAMO et al., 2020).

Uma variedade de substâncias naturais e artificiais são consideradas DEs, incluindo produtos farmacêuticos, produtos químicos indústrias, drogas, pesticidas, plastificantes e outros materiais comuns no dia a dia (LUCACCIONI et al., 2020).

A absorção dos DEs pode ocorrer por via oral (ingestão de água e alimentos), via respiratória (inalação de poeira, gases e partículas de ar), via dérmica (absorção cutânea) (INGARAMO et al., 2020), e também pode ocorrer durante a vida fetal e neonatal através da placenta e amamentação (CARGNELUTTI et al., 2021).

São capazes de afetar diferentes períodos da vida (vida pré-natal, pós natal e puberdade). Além disso podem ser compostos de diferentes combinações de produtos químicos (misturas), dificultando ainda mais a análise da sua ação na saúde humana (LUCACCIONI et al., 2020).

Os DEs estão associados a alterações reprodutivas, aumento da incidência de cancer de mama, padrões anormais de crescimento e atraso no desenvolvimento neurológico em crianças, bem como alterações na função imunológica (MONNERET, 2017).

Pesquisas relacionadas com regulação endócrina são descritas há anos. Alguns estudos de 1930 já demonstravam propriedades estrogênicas de produtos químicos industriais, dentre eles o DDT (diclorodifeniltricloroetano), um praguicida que induziu a feminilização em galos (ROMANO; ROMANO; OLIVEIRA, 2009).

Durante a vida, existem algumas fases (período crítico de desenvolvimento) que são extremamente importantes, pois, nelas transcorre uma alta atividade celular e a probabilidade de ocorrer alterações epigenéticas é maior, ou seja são períodos mais vulneráveis aos DEs. Esses períodos são a vida fetal, a vida neonatal e a puberdade (GREENSPAN; LEE, 2019; LUCACCIONI et al., 2020). A puberdade representa um marco crucial na vida reprodutiva de uma pessoa (FUDVOYE; LOPEZ-RODRIGUEZ; PARENT, 2019). O sistema endócrino reprodutivo pode ser afetado pelo GBH, através da interferência no eixo hipotalâmico hipofisário-gonadal, o qual é controlado minusiosamente pela produção e liberação de hormônios em quantidades pequenas (ROMANO; ROMANO; OLIVEIRA, 2009).

Os dados dos últimos 20 anos sugerem uma possível atuação dos DEs nas alterações no período da puberdade (FUDVOYE; LOPEZ-RODRIGUEZ; PARENT, 2019).

3.3 Agrotóxicos

Segundo a Lei N° 7.802, de 11 de julho de 1989, são considerados agrotóxicos (BRASIL, 1989):

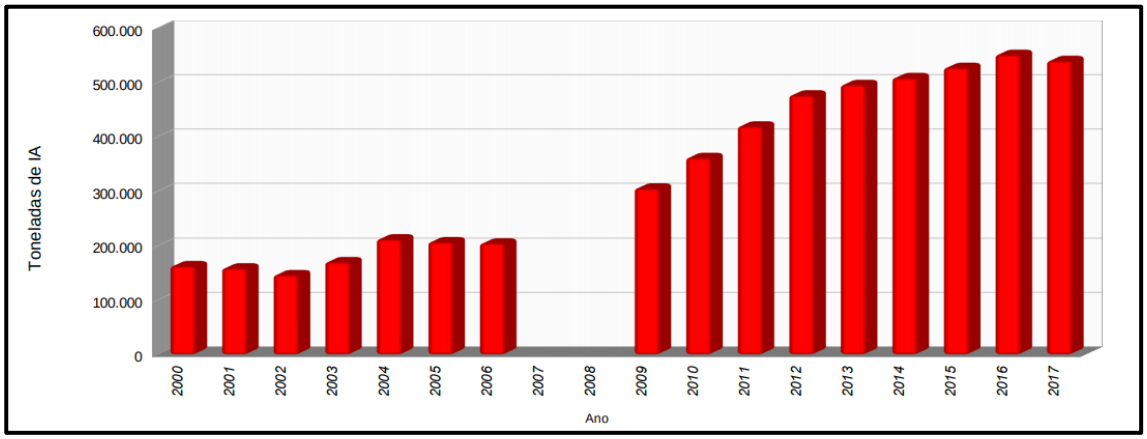
> Os produtos e os agentes de processos físicos, químicos ou biológicos, destinados ao uso nos setores de produção, no armazenamento e beneficiamento de produtos agrícolas, nas pastagens, na proteção de florestas, nativas ou implantadas, e de outros ecossistemas e também de ambientes urbanos, hídricos e industriais, cuja finalidade seja alterar a composição da flora ou da fauna, a fim de preservá-las da ação danosa de seres vivos considerados nocivos; substâncias e produtos, empregados como desfolhantes, dessecantes, estimuladores e inibidores de crescimento.

O aumento do uso dos agrotóxicos e sua toxicidade, torna a exposição da população a esses produtos um importante problema ambiental e de saúde pública (BRASIL, 2016).

Com a chegada do capitalismo e da modernização no campo pela “revolução verde”, fundamentada em estratégias para aumentar a produtividade através do incentivo do intenso uso de insumos químicos (agrotóxicos), biológicos (sementes melhoradas) e mecânicos (maquinário agrícola) (BRASIL, 2016; SERRA et al., 2016; TEJERINA, 2018), o paradigma hegemônico do setor agrícola brasileiro é marcado pela perversidade no modo com que explora o meio ambiente e a força de trabalho (ABRASCO, 2015). Desde então, as crescentes vendas e o uso de pesticidas no país têm gerado efeitos nocivos à saúde humana e prejuízos ambientais a curto, médio e longo prazo (BRASIL, 2016).

A Figura 1 apresenta as vendas de agrotóxicos e afins no Brasil no período de 2000 a 2017, expressas em toneladas de ingrediente ativo (IA) de acordo com o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), a quantidade desses produtos comercializados no ano de 2000 foi 162.461 toneladas, esse número elevou-se para 539.944 toneladas em 2017 (IBAMA, 2019).

Figura 1 - Vendas de agrotóxicos e afins no Brasil no período de 2000 a 2017



IA: Ingrediente Ativo. Fonte: IBAMA (2019). Disponível em: <http://www.ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos> (Acesso em 18/03/2019).

Segundo os dados do Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX), em 2016 no Brasil foram registrados 4.515 casos de intoxicação humana por agrotóxicos de uso doméstico e agrícola, desses, 101 foram a óbito, resultando em uma taxa de letalidade de 3,71% por agrotóxicos (FIOCRUZ, 2019).

Segundo os dados supracitados, a intoxicação humana por agrotóxicos, ficou atrás apenas aos medicamentos, agentes domissanitários e animais peçonhentos/venenosos. O SINITOX também notificou 2.737 casos de intoxicação humana por agente tóxico em circunstâncias ocupacionais em 2016 no Brasil, dentre esses, 815 foram por agrotóxicos (FIOCRUZ, 2019).

Embora sejam comercializados mais de 80.000 produtos químicos, os riscos deles ainda não foram submetidos à avaliação. Os agroquímicos estão entre as substâncias químicas que mais contaminam o meio ambiente e que não foram completamente avaliados (INGARAMO et al., 2020).

Atualmente, a maioria dos pesticidas atua como DE e seus efeitos deletérios são exercidos através da alteração do eixo hipotalâmico-hipofisário ou das ações que modificam a regulação de outros sistemas orgânicos, como o sistema

imunológico e o sistema nervoso (ESPINOZA-NAVARRO; PONCE-LAROSA; BUSTOS-OBREGÓN, 2017).

Entre as classes de pesticidas mais utilizadas no Brasil, encontra-se os inseticidas OFs. Os OFs possuem em sua composição um átomo de fósforo e no mínimo um de carbono (BARBOZA et al., 2018). Este tipo de inseticida atua inibindo a AChE, uma enzima responsável por hidrolisar o neurotransmissor ACh nas sinapses colinérgicas. A inibição desta enzima resulta no acúmulo de ACh dentro da sinapse (ESPINOZA-NAVARRO; PONCE-LAROSA; BUSTOS-OBREGÓN, 2017).

Enquadra-se na classe dos OFs, o glifosato, que é amplamente utilizado no Brasil (BRASIL, 2016), ocupando a primeira posição dos 10 ingredientes ativos mais vendidos no país no ano de 2017, correspondendo a 173.150,75 toneladas vendidas (IBAMA, 2019).

O glifosato é um herbicida pós emergente, não seletivo, de ação sistêmica (GIAQUINTO et al., 2017; AMARANTE JUNIOR et al., 2002), empregado mundialmente (SAMANTA et al., 2018). O mecanismo de ação do glifosato atua inibindo a enzima EPSPS (5-enolpiruvato-chiquimato-3-fosfato sintase) da via metabólica do ácido chiquímico em plantas, impedindo a síntese de determinados aminoácidos essenciais (triptofano, fenilanina e tirosina) ao crescimento das plantas. A via biossintética do ácido chiquímico não ocorre em vertebrados, pressupondo que o glifosato é seguro para mamíferos, incluindo os humanos (MESNAGE et al., 2015). Contudo, ao longo do tempo, estudos demonstram os efeitos prejudiciais provocados pelos herbicidas à base de glifosafo em diversas espécies, inclusive no ser humano (BAI; OGBOURNE, 2016).

Soudani et al., (2019), citaram que a exposição ao glifosato pode causar estresse oxidativo afetando o sistema nervoso, sistema reprodutivo e órgãos como fígado e rins, outros estudos sugerem que o glifosato pode ocasionar parkisionismo (ERIGUCHI et al., 2019; SAMSEL; SENEFF, 2015), autismo, doença de Alzheimer, transtorno de ansiedade, osteoporose, doença inflamatória intestinal, litíase renal, osteomalácia, colestase, disfunção tireoidiana (SAMSEL; SENEFF, 2015), neoplasias (SCHIMPF et al., 2017), efeitos carcinogênicos (GUYTON et al., 2015) e modificações no sistema endócrino (OWAGBORIAYE et al., 2019).

A pesquisa desenvolvida por Tizhe et al. (2014), observou as seguintes alterações histopatológicas em ratos *Wistar* expostos ao glifosato por oito semanas:

degeneração de células epiteliais da camada muscular da mucosa e das glândulas do estômago, degeneração das células hepáticas, especialmente nas áreas portais do fígado, degeneração glomerular nos rins, infiltração de células mononucleares nos interstícios dos túbulos e necrose tubular, degeneração neuronal no cérebro, degeneração de células acinares pancreáticas e despovoamento de células esplênicas nas polpas vermelha e branca.

3.4 Obesidade

A obesidade é definida como excesso de gordura corporal (NEWBOLD et al., 2008), decorrente da falta de equilíbrio orgânico, onde a ingestão calórica é maior que o gasto energético, comprometendo a saúde de um organismo (ABESO, 2016). Sua etiologia é multifatorial e envolve diversos fatores ambientais e genéticos (WANDERLEY; FERREIRA, 2010).

A obesidade e o sobrepeso impactam negativamente na saúde e prognósticos de uma série de doenças, como diabetes tipo 2, hiperinsulinemia, resistência à insulina, doença cardíaca coronária, hipertensão arterial, acidente vascular cerebral, gota, doença hepática, asma e problemas pulmonares (NEWBOLD et al., 2008). Estudos também evidenciaram efeitos negativos como atrofia muscular (YAN et al., 2021), fibras pequenas e arredondada e fibras com tamanho aumentado, caracterizando degeneração muscular, aumento do acúmulo de gordura e recrutamento de macrófragos na musculatura esquelética de animais obesos (ASGHAR et al., 2021). Rasslan et al., (2009) afirmam que a infiltração de gordura no diafragma e hipertonia dos músculos abdominais causados pela obesidade prejudicam o sistema respiratório através do comprometimento da função muscular e expansão torácica.

As comorbidades e complicações médicas associadas à obesidade tornam-a um importante problema de saúde pública (OLIVEIRA et al., 2021). O crescimento global dessa doença tem gerado preocupação, pois afeta crianças e adultos de todos os grupos socioeconômicos (NEWBOLD et al., 2008).

Tendo em vista que a obesidade tornou-se uma epidemia, além das causas genéticas, as ambientais também têm sido consideradas responsáveis (BAILLIE-HAMILTON, 2002), pois, o aumento dramático na incidência da obesidade ocorreu em paralelo com o aumento significativo no uso de DEs. Portanto, produtos químicos DEs têm sido associados com a obesidade em humanos (BOUDALIA et al., 2021).

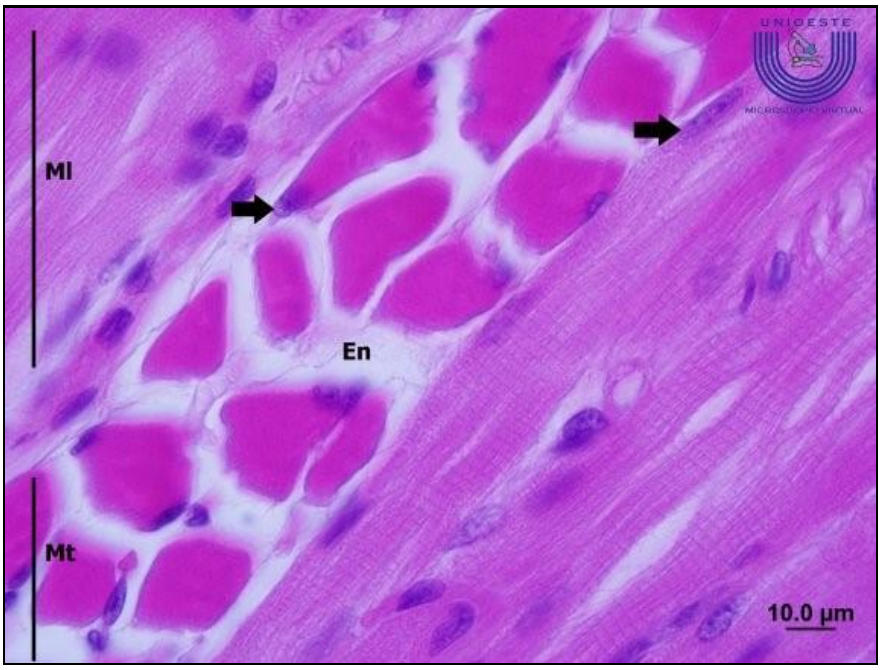
Nesse sentido, a identificação de substâncias obesógenas e a possível associação com os pesticidas, como o glifosato, com o desenvolvimento da obesidade, tem sido uma área em ascensão no campo científíco (NEWBOLD et al., 2008).

3.5 Músculo estriado esquelético e JNMs

O músculo estriado esquelético compreende 40% a 50% da massa corporal (KUNZ et al., 2013), é responsável pela geração de força para locomoção e movimentação corporal, sustentação postural e respiração (diafragma e músculos intercostais) (BIOLO; CEDERHOLM; MUSCARITOLI, 2014; DAL-PAI-SILVA; CARVALHO, 2007). Além disso, várias funções metabólicas como a homeostase energética, termorregulação, sensibilidade à insulina e metabolismo de aminoácidos estão envolvidas com o músculo esquelético (BIOLO; CEDERHOLM; MUSCARITOLI, 2014).

Este músculo é formado por células denominadas fibras musculares, caracterizadas pelo formato alongado, estreito e multinucleadas, com os núcleos localizados na periferia das fibras, próximo ao sarcolema (Figura 2). O tamanho da fibra depende da estrutura muscular, seu diâmetro pode variar de 10 a 100 µm e o comprimento pode alcançar até 10 cm (KUNZ et al., 2013). Essas fibras possuem contração rápida e forte, e são controladas voluntariamente (JUNQUEIRA; CARNEIRO, 2013).

Figura 2 - Fotomicrografia da estrutura do músculo estriado esquelético. Vista transversal (Mt) e longitudinal (Ml). Hematoxilina-Eosina (HE). Fibras multinucledas com núcleos em posição periférica (seta). Endomísio (En)

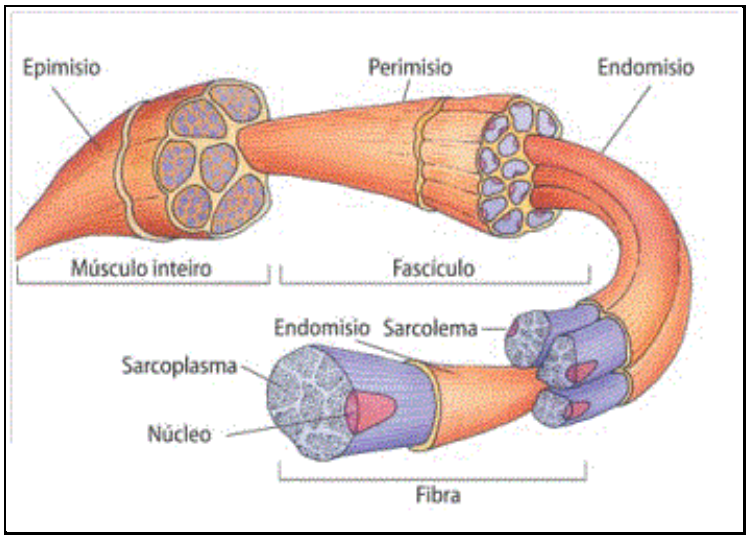


Fonte: Brancalhão et al., (2016). Disponível em: <http://projetos.unioeste.br/projetos/microscopio/> (Acesso em 27/03/2019)

O sarcômero é a unidade funcional da fibra muscular, e a repetição dessas unidades formam um sistema de estriações transversais pela existência de bandas claras e escuras dispostas longitudinalmente e alternadamente. Os filamentos grossos são formados de miosina, enquanto os filamentos finos são formados, em sua maior parte por actina (JUNQUEIRA; CARNEIRO, 2013; SILVERTHORN, 2010). A interação entre esses dois filamentos gera a contração muscular (JUNQUEIRA; CARNEIRO, 2013; MARQUES JUNIOR, 2015).

O tecido muscular é envolvido por uma matriz extracelular abundante em carbidratos e proteínas, o tecido conjuntivo, altamente organizado em: epimísio, responsável por recobrir todo o músculo; perimísio, que envolve um conjunto de fibras (fascículo muscular); e o endomísio, que recobre individualmente cada fibra muscular. O tecido conjuntivo transmite a força de contração gerada por cada fibra para todo o músculo (Figura 3) (DAL-PAI-SILVA; CARVALHO, 2007; OLARTE et al., 2017).

Figura 3 - Estrutura do músculo estriado esquelético



Fonte: Gartner; Hiatt (2007)

As fibras musculares podem ser classificadas de acordo com as suas características morfofuncionais. As fibras tipo I (lentas) possuem metabolismo oxidativo e são altamente resistentes à fadiga. As fibras do tipo IIb (rápidas) utilizam predominantemente o metabolismo glicolítico para a produção de energia, sendo mais rápidas, porém mais fatigáveis. Além dessas, existe um tipo intermediário de fibra, o tipo IIa, com metabolismo glicolítico-oxidativo, que contém características intermediárias (TALBOT; MAVES, 2016).

Considerado o principal músculo do sistema respiratório, o diafragma é inervado pelos nervos frênicos e composto por musculatura estriada esquelética, com particularidades próprias por possuir maior quantidade de fibras tipo I e, portanto, mais resistente à fadiga (SILVA, 2012).

Mesmo sendo um músculo esquelético, não é totalmente voluntário, pois a respiração acontece através do comando do centro respiratório no bulbo, que ao tentar efetuar uma contração muscular, promove uma diferença de pressão torácica (SANTOS-JÚNIOR et al., 2010).

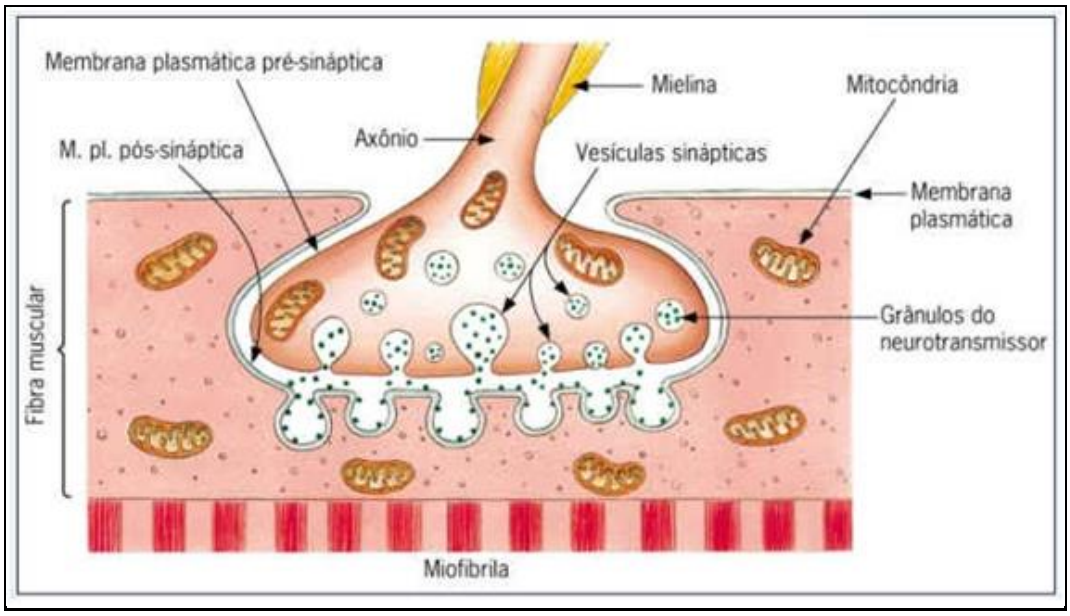
O contato com os agrotóxicos pode ocorrer através das mucosas, pele, respiração ou ingestão (NEVES; BELLINI, 2013), essa exposição pode desencadear diferentes problemas respiratórios, além disso, alguns compostos como os OFs

podem provocar alterações musculares, diminuição de força e disfunção motora (SOARES; ALMEIDA; MORO, 2003).

Cada fibra muscular esquelética recebe individualmente os impulsos nervosos provenientes de um neurônio motor em uma região sináptica, chamada junção neuromuscular (JNM). Neste local, o potencial de ação de um nervo induz a liberação de um neurotransmissor, a ACh, que age sobre a membrana da fibra muscular, iniciando um potencial de ação (TORREJAIS et al., 2009) e gerando a contração do músculo (FAGERLUND; ERIKSSON, 2009).

A JNM é estruturada basicamente por um terminal pré-sináptico, que consiste no neurônio motor, com seu corpo celular localizado no corno anterior da medula espinal e inerva a fibra muscular atrávés de longas extensões (axônios mielinizados) (FAGERLUND; ERIKSSON, 2009), a ACh é sintetizada no corpo do neurônio, armazenada no terminal pré sinápticos e liberada na fenda sináptica, que compreende uma matriz extracelular estrutural e funcional entre os dois terminais, e, por fim, o terminal pós-sináptico, que corresponde a membrana sarcoplasmática, e contém receptores de ACh e outras moléculas importantes no metabolismo da JNM (JUNQUEIRA; CARNEIRO, 2013; PUNGA; RUEGG, 2012) (Figura 4).

Figura 4 - Estrutura da junção neuromuscular



Fonte: Disponível em: <http://www.museuescola.ibb.unesp.br/subtopico.php?id=2&pag=2&num=3&sub=23> (Acesso em 17/06/2019).

3.6 Efeito dos agrotóxicos organofosforados no músculo estriado esquelético

A neurotoxicidade dos OFs se deve, entre outros fatores, pelo mecanismo de ação de inibição da AChE, enzima responsável pela degradação da ACh, ocorrendo, portanto, o aumento da concentração desse neurotransmissor dentro das sinapses (BARBOZA et al., 2018; SILVA; CAMPOS; BOHM, 2013). Em função desse acúmulo, os receptores nicotínicos são hiperestimulados nas sinapses do sistema nervoso central, sistema nervoso autônomo e JNMs (CAVALCANTI et al., 2016; MOON; CHUN; LEE, 2015), modificando a permeabilidade do cálcio (Ca^{2+}) nas células.

Com uma maior quantidade de Ca^{2+} intracelular, haverá maior ativação de proteínas denominadas calpaínas e ativação de quinases Ca^{2+}/dependentes (FERNANDES, 2017), capazes de promover o desarranjo das proteínas do citoesqueleto e desorganização sarcomérica (FERNANDES, 2017; HOLOVSKÁ et al., 2017). Estudos comprovam que a alta concetração intracelular de Ca^{2+} danifica as mitocôndrias e a contração anormal das miofibrilas causada pelo acúmulo de ACh pode favorecer o dano à fibra muscular (HOLOVSKÁ et al., 2017).

Os efeitos causados por esse processo de hiperestimulação colinérgica decorrente das intoxicações por compostos OFs variam conforme a gravidade,

velocidade de instalação, dose, período de tempo e meio de exposição (CAVALCANTI et al., 2016).

Sintomas como hiperatividade simpática e disfunção neuromuscular, taquicardia, hipertensão, fasciculações musculares e até paralisia se manifestam em intoxicações por OFs (BRASIL, 2015), podendo resultar em falência respiratória e consequentemente, morte (CAVALCANTI et al., 2016; SOUZA; GONÇALVES, 2019).

Diante disso, estudos exploram os efeitos da exposição à compostos OFs em animais. Uma pesquisa realizada com ratos, observou que a administraçao do composto OF Sarin causou alterações miopáticas (degeneração muscular e infiltrado mononuclear) no músculo diafragma desses animais (BRIGHT et al., 1991).

Calore et al., (1999) investigaram o diafragma de ratos intoxicados com o OF Isofenphos, os resultados demonstraram alterações metabólicas como a inibição de lipase e esterase do sarcoplasma resultando no acúmulo de lipídios. Segundo os autores essas alterações devem contribuir para indução e progressão necrótica da fibra muscular, fraqueza muscular e comprometimento clínico de animais e humanos intoxicados agudamente por esses compostos.

A incidência de miopatia induzida por Paraoxon em diferentes tipos de fibras músculares de ratos. Músculo misto com predominância de fibras tipo I ou oxidativas como o diafragma e músculos puramente oxidativos como o masseter e o sóleo apresentaram necrose de forma mais extensa, bem como, a lesão de outros sete músculos mistos com predominância em fibras tipo II foram menores. Concluiu que músculos compostos predominantemente por fibras do tipo oxidativas são mais suscetíveis à miopatia necrosante induzida por OFs, contudo, a classificação oxidativa não é o único fator que interfere na vulnerabilidade do músculo esquelético (DE BLEECKER et al., 1992).

Segundo Cavaliere et al., (1996, p. 268):

> Alterações estruturais e funcionais observadas nos músculos esqueléticos estão relacionadas com as estruturas químicas dos produtos e com o tipo de músculo, sendo o diafragma o mais afetado em estudos experimentais. Necrose muscular, semelhante à observada em animais de laboratório, foi encontrada em músculos diafragma e intercostais de indivíduos intoxicados

> pelo parathion, diazinon, triclornato e combinação de malathion e diazinon. Uma vez que a insuficiência respiratória é uma das consequências mais graves das intoxicações por organofosforados, é possível que o envolvimento dos músculos da respiração constitua agravante do distúrbio respiratório.

Tendo em vista que os agrotóxicos e a obesidade são considerados um problema de saúde pública atual e possuem efeitos deletérios sobre o músculo estriado esquelético, e que o glifosato ainda pode atuar como DE, torna-se necessário compreender a organização e comportamento das fibras musculares do diafragma de camundongos adultos, obesos ou não, expostos ao GBH durante o período peripuberal e qual a sua relação sobre sistema muscular estriado esquelético e suas respectivas funções.

ARTIGO CIENTÍFICO 1

EFEITOS DA EXPOSIÇÃO PERIPUBERAL AO HERBICIDA À BASE DE GLIFOSATO NO MÚSCULO DIAFRAGMA DE CAMUNDONGOS ADULTOS

REVISTA: TISSUE AND CELL

NORMAS DA REVISTA: ANEXO 02

EFEITOS DA EXPOSIÇÃO PERIPUBERAL AO HERBICIDA À BASE DE GLIFOSATO NO MÚSCULO DIAFRAGMA DE CAMUNDONGOS ADULTOS

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RESUMO

Introdução: O glifosato é um herbicida organofosforado com efeitos sobre diversos sistemas, incluindo o sistema respiratório. O objetivo dessa pesquisa foi analisar as fibras musculares e junções neuromusculares (JNMs) do músculo diafragma de camundongos adultos expostos ao herbicida à base de glifosato no período peripuberal.

Métodos: Foram utilizados doze camundongos machos, separados em grupo controle (CTL, n=6), que recebeu água, e grupo herbicida à base de glifosato (GBH, n=6), que recebeu 50 mg/Kg/dia de Roundup®, ambos por gavagem dos 30 aos 60 dias. Aos 150 dias os animais foram eutanasiados e o diafragma coletado para análise das fibras musculares através das colorações hematoxilina-eosina, tricrômicro de Masson e Picrossirius Red e das JNMs através da reação de esterese inespecífica.

Resultados: Foram evidenciados fibras arredondadas, sarcoplasma hipereosinofílico e núcleos aumentados predominantemente no GBH. Foi observado, também, a redução no peso corporal, aumento na morfometria das fibras musculares, aumento do colágeno tipo III, diminuição da sobreposição do colágeno tipo I e III, e aumento na área e diâmetro maior das JNMs no GBH.

Conclusão: A exposição peripuberal ao GBH evidenciou alterações morfológicas características de degeneração muscular e alterou a morfometria das fibras musculares e JNMs do diafragma de camundongos adultos.

Palavras-chave: Glifosato; Puberdade; Fibras Musculares Esqueléticas; Junções Neuromusculares.

1. Introdução

O glifosato ou N- (fosfonometil) glicina, é um pesticida organofosforado, de ação sistêmica, não seletivo e de amplo espectro (Pandey et al., 2019; Reynoso et al., 2019), que constitui o principal princípio ativo contido no Roundup® (Owagboriaye et al., 2019). Os herbicidas à base de glifosato são amplamente utilizados no mundo (Gasnier et al., 2009), entretando, seu uso exacerbado na agricultura tem deixado resíduos no solo, água e alimentos (Gill et al., 2018; Pandey et al., 2019).

A literatura mostra esse herbicida como um potencial disruptor endócrino (Gasnier et al., 2009; Gomez et al., 2020; Mesnage et al., 2015; Romano et al., 2009). Os disruptores endócrinos são substâncias ou misturas exógenas que alteram as funções do sistema endócrino e, consequentemente, causam efeitos deletérios à saúde do organismo ou da sua prole (Who/Unep, 2013). A exposição a xenobióticos durante os momentos críticos do desenvolvimento e maturação, pode estar relacionada à alteração do padrão de expressão gênica, uma vez que é durante esses períodos em que o organismo estabelece os principais padrões epigenéticos, períodos esses organizados em vida fetal, vida neonatal e puberdade (Greenspan and Lee, 2019; Lucaccioni et al., 2020). Sendo que, as consequências dessa exposição precoce a herbicidas à base de glifosato podem se manifestar apenas em estágios posteriores ao desenvolvimento e/ou na fase adulta (Myers et al., 2016).

A puberdade é o período de crescimento infantil que precede o início da adolescência (Dutta and Sengupta, 2016), é um processo de crescimento físico, desenvolvimento emocional e grande plasticidade cerebral (Dutta and Sengupta, 2016). Nessa fase, ocorrem mudanças estruturais e funcionais que podem interferir no metabolismo na fase adulta (Blakemore et al., 2010; Pervanidou and Chrousos, 2012).

O contato com os agrotóxicos pode ocorrer através das mucosas, pele, respiração ou ingestão (Neves and Bellini, 2013) e essa exposição pode desencadear diferentes problemas respiratórios (Hoppin et al., 2014; Rathinam et al., 2005; Slager et al., 2010; Soares et al., 2003). O comprometimento da musculatura

respiratória, causado pela intoxicação por organofosforados, possivelmente contribui para o alto índice de morbidade em intoxicações (Cavaliere et al., 1996).

O diafragma é considerado o principal músculo do sistema respiratório (Silva et al., 2012). Esse músculo é inervado pelos nervos frênicos (Orozco-Levi and Servei, 1997) e composto por diferentes tipos de fibras, portanto, é classificado como um músculo misto (Rowley et al., 2005). Ele possui algumas particularidades próprias como maior resistência à fadiga, maior fluxo sanguíneo, maior capacidade oxidativa e uma maior densidade capilar. Essas propriedades permitem resistência necessária para as atividades de alta demanda ao longo da vida (Orozco-Levi and Servei, 1997).

Dessa forma, o músculo diafragma foi escolhido para este estudo, por estar intimamente ligado ao processo da respiração. Assim, o objetivo dessa pesquisa foi analisar as fibras musculares e junções neuromusculares (JNMs) do diafragma de camundongos adultos expostos ao GBH durante o período peripuberal.

2. Materiais e Métodos

2.1 Aspectos éticos

Os procedimentos adotados nessa pesquisa foram aprovados pelo Cômite de Ética em Uso de Animais (CEUA) da UNIOESTE em 2018, e todas as diretrizes institucionais para o cuidado e uso de animais foram aplicadas.

2.2 Procedimentos experimentais

2.2.1 Obtenção dos animais

Foram utilizados 12 camundongos machos, da linhagem C57BL/6 com 21 dias de idade e média de peso corporal (PC) de 20g. Os animais foram adquiridos do biotério central da Universidade Estadual do Oeste do Paraná - UNIOESTE e foram mantidos no biotério setorial do Laboratório de Fisiologia Endócrina e

Metabolismo - LAFEM, em condições controladas de temperatura (28 ± 2ºC) e luminosidade (12 horas claro/escuro).

2.2.2 Grupos experimentais

Aos 30 dias de idade ocorreu o desmame e, então, animais foram separados aleatoriamente em dois grupos, sendo que, apenas dois filhotes da mesma mãe foram alocados em um mesmo grupo: Grupo controle (CTL, n=6) e Grupo herbicida à base de glifosato (GBH, n=6). Todos os camundongos receberam água *ad libitum* e foram alimentados com dieta padrão para roedores (Nuvital, Brasil) a partir do desmame até os 150 dias de idade.

2.2.3 Administração do GBH

Dos 30 aos 60 dias de vida, diariamente por gavagem, o grupo GBH recebeu 50 mg/Kg de PC (Romano et al., 2008) da solução Roundup® Original DI (Monsanto, Brasil, contendo 445 g/L de sal de diamônio de N-fosfonometilglicina correspondente a 370 g/L ou 37,0% m/v, do componente ativo glifosato), e o grupo CTL recebeu água. A solução do GBH e a água tiveram o pH corrigidos entre 5 e 5,5 com ácido clorídrico.

2.2.4 Eutanásia e extração dos tecidos

Completados 150 dias de vida, os animais foram pesados e medidos para obtenção do comprimento nasoanal. Em seguida, os camundongos foram anestesiados com uma mistura de cloridrato de xilazina (Anasedan®, Vetbrands, Brasil) e cloridrato de cetamina (Dopalen®, Vetbrands, Brasil) nas concentrações 9 mg/Kg e 90 mg/Kg, respectivamente. Em seguida, foi realizada laparotomia para a coleta dos órgãos. As gorduras retroperitoneal e perigonadal foram coletadas e pesadas e padronizadas pelo peso (Borck et al., 2020). Para a coleta do músculo diafragma, os animais foram mantidos em posição ventral e uma incisão na região mediana foi realizada logo abaixo do tórax, com o posterior rebatimento da pele e dos músculos. A retirada do diafragma consistiu apenas em sua parte costal, sendo

dividida em antímero direito e esquerdo. Apenas o antímero direito foi pesado. Em seguida, cada antímero foi fragmentado em dois segmentos, sendo que no antímero direito o fragmento superior e inferior foram destinados para processamento histológico em parafina. Já no antímero esquerdo, os segmentos superior e inferior foram destinados a estudo histoquímico das JNMs. Em seguida, os fragmentos foram armazenados em fixadores específicos para realização do estudo histológico e histoquímico.

2.2.5 Estudo histológico das fibras musculares

Os fragmentos do antímero direito do músculo diafragma foram fixados em *Methacarn,* e em seguida foram destinados ao processamento histológico para inclusão em parafina. O músculo diafragma foi seccionado transversalmente com cortes semi-seriados a sete µm de espessura, foram aplicados seis cortes em cada lâmina e foram utilizadas duas lâminas por animal para cada coloração. As lâminas foram submetidas à técnica de coloração Hematoxilina-Eosina (HE), conforme técnica de Junqueira e Junqueira (1983), e coloração de Tricrômico de Masson (Bancroft and Steven, 1990). As lâminas coradas pela técnica de HE foram utilizadas para quantificação das fibras musculares, núcleos, capilares, relação capilar/fibra e relação núcleo/fibra, a partir da análise de dez campos microscópicos (objetiva de 40x) para cada animal. O material corado através da técnica de Tricômico de Masson foi utilizado para quantificar o tecido conjuntivo. A análise foi realizada através de dez campos microscópicos para cada animal (objetiva de 20x). Parte das lâminas foram destinadas para a coloração de Picrossirius Red (Junqueira et al., 1979), para a verificação do padrão de qualidade da coloração e a confirmação da refringência das fibras colágenas. Para essa análise foram utilizados oito campos por animal (objetiva de 40x). De cada campo foi quantificado o percentual de pixels referente ao colágeno do tipo I (pixels de cor verde), colágeno do tipo III (pixels de cor vermelha) e áreas de sobreposição de colágeno I e III (pixels de cor amarela).

2.2.6 Estudo morfológico e morfométrico das JNMs

Os fragmentos superiores e inferiores do antímero esquerdo do diafragma foram fixados em Karnovsky (Karnovsky, 1965) em temperatura ambiente, os fragmentos foram seccionados longitudinalmente em várias fatias finas com lâminas de inox, e os cortes obtidos foram submetidos à reação Esterase Inespecífica (Lehrer and Ornstein, 1959). A análise morfométrica das JNMs foi realizada através das medidas de área, diâmetros maior e menor de 100 JNMs por animal estudado, obtidas a partir de imagens microscópicas (objetiva de 20x).

2.3 Análise das imagens

As imagens das fibras musculares e JNMs foram obtidas através do microscópio Olympus Bx60® acoplado à câmera Olympus DP71 (Tókio, Japão), com auxílio do programa DP Controler 3.2.1 276. Para análise morfométrica das fibras musculares e JNMs foi utilizado o programa Image Pro Plus 6.0® (Media Cybernetics, Maryland, USA). Para a mensuração do tecido conjuntivo foi utilizado o Programa de Manipulação de Imagens GNU - GIMP 2.10.22 (GNU General Public License®, Berkeley, Califórnia, Estados Unidos), e para a obtenção da área relativa do tecido conjuntivo (densidade da área) foi dividido o total de pixels da fotomicrografia pelo total de pixels da marcação do tecido conjuntivo. As imagens das fibras colágenas do tecido conjuntivo obtidas através da coloração de picrossirius red foram visualizadas no microscópio de luz polarizada (campo escuro), modelo Axio Imager A1 (Carl Zeiss, Alemanha), acoplado à uma câmera digital modelo Axiocam MRc (Zeiss Vision, Alemanha). E para a mensuração das estruturas visualizadas no tecido conjuntivo, foi utilizado o Programa de Manipulação de Imagens GNU - GIMP 2.10.30.

2.4 Análise estatística

Os dados obtidos foram submetidos à análise estatística através do programa GraphPad Prism® (La Jolla, USA). Foi analisada a normalidade dos dados pelo teste de Teste *Kolmogorov-Smirnov*. Para os dados que se encontravam em normalidade, o teste estatístico empregado foi teste *t* de Student, para os dados que não estavam

em normalidade foi utilizado o teste *Mann-Whitney*. Valores de $p < 0,05$ foram considerados com significância estatística.

3. Resultados

3.1 Parâmetros macroscópicos

Foi observado que o grupo GBH apresentou redução significativa do PC ($p = 0,0174$) quando comparado ao grupo CTL (Tabela 1). Contudo, os dados de comprimento nasoanal ($p = 0,3468$), peso das gorduras retroperitoneal ($p = 1,0000$) e perigonadal ($p = 0,9682$), e peso do músculo diafragma ($p = 0,2265$) não demonstraram diferença estatística quando realizada a comparação entre os grupos (Tabela 1).

3.2 Análise morfológica e morfométrica das fibras musculares

A morfologia da maioria das fibras musculares apresentou arquitetura geral preservada. Estas possuem aspecto poligonal, multinucleadas com seus núcleos periféricos e organizadas em fascículos, em ambos os grupos estudados (Figuras 1A e 1B). Contudo, foram observadas fibras com núcleos aumentados e alguns com halo basofílico em maior número no grupo GBH (Figuras 1C e 1D). Foram encontrados também núcleos centrais (Figuras 1E e 1F) nos dois grupos de maneira equivalente e fibras com formato arrendondado e sarcoplasma hipereosinofílico (Figuras 1G e 1H) nos grupos CTL e GBH, mas predominantemente no grupo GBH.

Em relação a morfometria das fibras musculares coradas com HE, foi verificado aumento significativo na área das fibras musculares ($p = 0,0112$), diâmetro maior ($p = 0,0148$) e diâmetro menor ($p = 0,0174$) do grupo GBH quando comparado ao grupo CTL. Quando realizada a contagem do número de fibras musculares, não foi constatada diferença significativa na densidade das fibras entre os dois grupos estudados ($p = 0,3078$) (Tabela 2).

Não houveram diferenças significativas nos grupos estudados em relação ao número de núcleos periféricos ($p = 0,5051$), número de núcleos centrais ($p =$

0,2141), relação núcleo/fibra (p = 0,9503), número de capilares (p = 0,5885) e relação capilar/fibra (p = 0,7354) (Tabela 2).

3.3 Análise de tecido conjuntivo intramuscular e quantificação de colágenos tipo I e III

Na análise do tecido conjuntivo das fibras musculares do diafragma coradas através da coloração de Tricrômico de Masson, foi observado esse tecido predominantemente no perimísio, membrana que envolve e delimita os fascículos de fibras musculares (Figuras 2A e 2B). Quando avaliada a porcentagem de tecido conjuntivo intramuscular, não houve diferença significativa entre os grupos GBH e CTL (Tabela 3).

A coloração do Picrosirius Red permitiu identificar e quantificar as fibras colágenas tipo I e tipo III e a sobreposição desses dois tipos de colágeno na matriz extracelular do diafragma. Os colágenos tipo I e III apresentaram-se com marcação mais abundante no perimísio (Figura 3A e 3B). Não houve diferença significativa entre os grupos CTL e GBH com relação à quantidade de colágeno tipo I. Contudo, foi evidenciado um aumento na quantidade de colágeno tipo III e consequentemente, diminuição da sobreposição do colágeno tipo I e tipo III no grupo GBH (Tabela 3).

3.4 Análise morfológica e morfométrica das JNMs

As JNMs encontradas no músculo diafragma dos animais estudados apresentaram-se polimórficas, com forma oval, redonda ou elíptica (Figuras 4A e 4B).

A análise morfométrica evidenciou aumento significativo na área (p = 0,0264) e diâmentro maior (p = 0,0044) das JNMs do grupo GBH quando comparado ao CTL (Figuras 4C e 4D). Em relação ao diâmetro menor das JNMs, não foi observada diferença significativa (p = 0,1049) entre os dois grupos estudados (Figura 4E).

4. Discussão

Este estudo demonstrou que a exposição ao GBH na fase peripuberal dos camungos resultou em menor PC na fase adulta. Os achados deste estudo corroboram com os resultados encontrados na literatura, de que a exposição ao glifosato pode reduzir o PC. Um estudo mostrou redução no PC de ratos machos que receberam 500 mg/kg de glifosato por 35 dias (Tang et al., 2017). Os resultados de Bali et al., (2017) mostraram que a exposição subcrônica e crônica ao GBH (nas doses de 250 e 500 mg/kg) diminuiu o ganho de PC de camundongos tratados desde a idade juvenil até a adulta.

Mudanças no PC podem estar relacionadas a diversos fatores como acúmulo reduzido de proteínas e lipídios, que desempenham papel fundamental nas reservas de energia (Ren et al., 2018), ou pelo resultado de elevado consumo energético a fim de desintoxicar o organismo (Milesi et al., 2021), por menor consumo água causado por alteração do sistema nervoso (regulador do teor hídrico) (Barbosa et al., 2021), por alterações decorrentes do estresse oxidativo no organismo causado por inseticidas organofosforados (Bali et al., 2017; Jasper et al., 2012; Selmi et al., 2018), ou então, pelo efeito desregulador endócrino desse herbicida (Pandey and Rudraiah, 2015).

O aumento do volume nuclear encontrado nos animais expostos ao GBH pode estar relacionado com o efeito tóxico dos organofosforados ao tecido, uma vez que, como consequência da toxicidade celular promovida pelo GBH, pode haver uma maior expressão de proteínas do sistema de defesa antioxidante e de reparo (Osuna-Flores et al., 2019). Dessa forma, os núcleos com volume aumentado podem indicar um aumento na taxa de síntese proteica (Santos et al., 2019), bem como relacionados ao processo de regeneração da fibra muscular (Crabbs, 2021).

Foram notadas também, fibras arredondadas e hipereosinofílicas nos dois grupos estudados, mas com ênfase no grupo GBH. O arredondamento das fibras musculares pode ser característica de miopatia (Camargo Filho et al., 2006) e as fibras hipereosinofílicas indicam degeneração muscular (Sihvo et al., 2013). Alterações semelhantes já foram descritas na musculatura estriada esquelética após o uso produtos miotóxicos (Greaves et al., 2013). Esses resultados demonstram que a fibra muscular do diafragma pode ter sofrido algum tipo de lesão e/ou degeneração após a administração do GBH.

Os dados resultados deste estudo mostraram um aumento na área, diâmetro maior e menor na análise das fibras musculares do diafragma, importante músculo respiratório.

Qualquer mudança nas demandas funcionais pode fazer o tecido muscular responder de forma diferente, alterando seus aspectos estruturais, metabólicos e funcionais (Aguiar and Aguiar, 2009; Van der Meer et al., 2011).

Estudos mostraram que a exposição ao glifosato induz a alterações nos biomarcadores de estresse oxidativo em peixes (Glusczak et al., 2011; Moura et al., 2017; Teixeira et al., 2018) e em ratos (Ren et al., 2018; Tang et al., 2017; Turkmen et al., 2019). Os resultados de Possamai et al., (2007) indicaram que a exposição ao organofosforado Malathion pode estar diretamente envolvida nos danos oxidativos nos músculos diafragma e quadríceps em ratos. Os organofosforados produzem estresse oxidativo em diferentes tecidos através da formação de espécies reativas de oxigênio, que quando produzidas em excesso, causam lesões nos tecidos, incluindo peroxidação lipídica, modificações no DNA e inativação enzimática (Possamai et al., 2007). Deste modo, o estresse oxidativo causado pelo GBH também poderia explicar as alterações morfométricas nas fibras musculares encontradas neste estudo.

Esse estudo não analisou os níveis hormônais dos animais estudados, mas, outros autores evidenciaram os efeitos de disrupção endócrina do GBH. Nardi et al., (2017) administrou o GBH (50 e 100 mg/kg) em ratos machos durante o período peripuberal. A disrupção endócrina foi observada por meio da redução dos níveis de testosterona, diminuição do número de células de Sertoli e aumento da degeneração das células de Sertoli e Leydig em ambas as doses. Romano et al., (2012), também demonstrou que a exposição de ratos machos ao GBH durante a puberdade também reduz a produção de testosterona. Outra explicação é que o GBH no período peripuberal possa ter promovido a alterações morfométricas nas fibras musculares do diafragma de camundongos adultos através da disrupção endócrina, considerando que a testosterona regula o metabolismo de proteínas musculares (Bhasin et al., 2001).

Tendo em vista que a intoxicação por organofosforados pode causar insuficiência respiratória (Gaspari and Paydarfar, 2012; Klein-rodewald et al., 2011) e

que a força e contração do diafragma aumenta progressivamente em decorrência da carga de trabalho imposta sobre ele (Kabitz et al., 2007), é possível que uma disfunção respiratória causada pela exposição ao GBH tenha gerado uma sobrecarga sobre esse músculo, resultando assim na hipertrofia das suas fibras, pois, o recrutamento das unidades motoras do diafragma varia de acordo com comportamento ventilatório. Uma maior frequência respiratória vai aumentar o recrutamento de unidades motoras, resultando em exagerada ativação do diafragma (Sieck et al., 2012), contudo, levando consideração essa possível explicação e os efeitos sistêmicos dos agrotóxicos sobre o organismo, provavelmente essa hipertrofia do diafragma não seja benéfica.

Além disso, segundo alguns autores, o glifosato pode promover alterações funcionais cardíacas importantes (Ros et al., 2020; Souza et al., 2017; Turkmen et al., 2019). A função metabólica e contrátil do músculo diafragma está associada ao perfeito funcionamento da circulação sanguínea (Silva et al., 2005). Silva et al., (2005), avaliaram o estresse oxidativo no músculo diafragma de ratos após uma insuficiência cardíaca induzida. Eles relataram aumento (217%) da lipoperoxidação no diafragma, redução da atividade antioxidante da catalase (77%) e da glutationa peroxidase (20%) dos animais infartados, confirmando, assim, a presença de estresse oxidativo no diafragma após a lesão cardíaca.

Portanto, uma explicação para as alterações morfométricas das fibras do diafragma podem ser resultantes de congestão pulmonar, decorrentes de alteração mecânica cardíaca e, consequentemente, redução do débito cardíaco, causado pelo uso do glifosato. Visto que, durante a insuficiência cardíaca, ocorre a congestão pulmonar e a sobrecarga do músculo diafragma (Silva et al., 2005), uma vez que suas fibras se contraem na tentativa de melhorar a congestão pulmonar, colaborando para o aumento no tamanho das fibras musculares do diafragma, pois o padrão de ativação contínua e repetitiva do diafragma pode torná-lo particularmente suscetível a adaptações (Prakash et al., 1995). Essa sobrecarga imposta à musculatura justificaria o aumento na área, diâmetro maior e menor das fibras do diafragma.

Outros experimentos já demonstraram que compostos organofosforados possuem efeitos deletérios diretos sobre os músculos esqueléticos, inclusive, o

diafragma (Bright et al., 1991; De Bleecker et al., 1992). De Bleecker et al., (1992), ainda afirmaram que músculos compostos por fibras mistas mas predominantemente oxidativas, estão mais propensos à lesão celular. O diafragma se encaixa nessa classificação do tipo de fibra, sendo assim, essa característica também o torna mais suscetível a alterações provocadas por organofosforados, como observamos nessa pesquisa.

A Matriz extracelular do tecido muscular é composta principalmente por colágeno tipo I e tipo III que circundam as fibras musculares e oferecem suporte, proteção e mantêm a sua integridade (Souza et al., 2011). No presente estudo, foi evidenciado um aumento no colágeno tipo III no tecido conjuntivo do grupo GBH. Há uma correlação entre o aumento na síntese de colágeno e o processo regenerativo que o ocorre no músculo esquelético, ou seja, um aumento na síntese de colágeno após algum tipo de sobrecarga sobre a musculatura pode indicar parte de um processo reparador (Kjaer et al., 2006; Souza et al., 2011), com ou sem dano tecidual evidente (Souza et al., 2011).

Diante dos resultados encontrados nesse estudo, reforça-se a ideia de que o músculo diafragma possa ter sofrido adaptações e/ou lesões decorrentes de uma sobrecarga após a exposição ao GBH, resultando no aumento da síntese de colágeno tipo III, na morfometria das fibras musculares e no tamanho dos mionúcleos.

Além disso, alguns autores já relataram um aumento na deposição de colágeno tipo III no fígado de ratos Wistar após a exposição ao glifosato Biocarb (Benedetti et al., 2004) e um maior nível de birrefrigência de colágeno glândulas mamárias de ratos machos expostos ao GBH no período pré puberal (Altamirano et al., 2018). Isto significa que, o glifosato, por si só, pode aumentar a quantidade colágeno em diferentes tecidos no organismo.

O GBH no período peripuberal alterou a área e diâmetro maior das JNMs do músculo diafragma em camundongos adultos. Gallegos et al., (2018) expôs ratas prenhas via oral ao GBH desde o início da gestação até o desmame. Foi observada alteração antioxidante e diminuição da acetilcolinesterase (AChE) no cérebro da prole adulta exposta ao GBH. Embora o estudo citado não tenha investigado a atividade da AChE no músculo esquelético, este reforça que, a exposição a GBH

durante estágios iniciais do desenvolvimento afeta os marcadores de estresse oxidativo e a atividade da AChE no sistema colinérgico a longo prazo. Uma possível explicação é que o GBH, sendo um composto organofosforado, possa ter inibido a enzima neuromuscular AChE, responsável por hidrolisar a acetilcolina (Barboza et al., 2018; Lajmanovich et al., 2011; Silva et al., 2013). Nesse caso, a acetilcolina permaneceria mais tempo na fenda sináptica, superestimulando os receptores colinérgicos nas JNMs (Cavalcanti et al., 2016; Moon et al., 2015). Esse estimulo excessivo pode ter aumentado morfometria das JNMs, e nesse caso, essas alterações nas JNMs poderiam justificar também as modificações encontradas na morfometria das fibras, pois elas poderiam estar sendo ativadas exageradamente também, pelo acúmulo da acetilcolina na JNM.

É possível que a investigação de outras estruturas possam nortear como os mecanismos de toxicidade do GBH ingerido no período peripuberal afetam o músculo na idade adulta. Sugere-se, portanto, a realização de novos estudos que avaliem outras estruturas associadas ao músculo diafragma, como, por exemplo, a atividade da enzima AChE, marcadores de estresse oxidativo e marcadores epigenéticos no diafragma de camundongos machos adultos expostos ao GBH no período peripuberal.

5. Conclusão

Diante dos resultados encontrados nessa pesquisa, conclui-se que a exposição peripuberal ao GBH altera a morfometria das fibras musculares do diafragma, resulta em alterações morfológicas características de degeneração muscular, e altera a morfometria das JNMs de camundongos machos adultos.

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Referências

Aguiar, A.F., Aguiar, D.H., 2009. Plasticidade muscular no exercício físico. R. Bras. Ci. e Mov. 17, 104–113.

Altamirano, G.A., Delconte, M.B., Gomez, A.L., Ingaramo, P., Bosquiazzo, V.L., Luque, E.H., Muñoz-de-toro, M., Kass, L., 2018. Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats. Food Chem. Toxicol. 118, 111–118. https://doi.org/10.1016/j.fct.2018.05.011

Bali, Y.A., Ba-mhamed, S., Bennis, M., 2017. Behavioral and Immunohistochemical Study of the Effects of Subchronic and Chronic Exposure to Glyphosate in Mice. Front. Behav. Neurosci. 11, 146. https://doi.org/10.3389/fnbeh.2017.00146

Bancroft, J.D., Steven, A., 1990. Theory and practice of histological techniques, 3rd ed. Churchill Livingstone, Edinburg.

Barbosa, A., Oliveira, M.C., Kuhn-fraga, C., Balbo, S.L., Torrejais, M.M., 2021. Estudo das fibras musculares dos músculos extensor longo dos dedos e sóleo de fêmeas C57BL / 6 expostas ao glifosato durante a prenhez e a lactação. Einstein (Sao Paulo) 19, 1–7. https://doi.org/10.31744/einstein_journal/2021AO5657

Barboza, H.T.G., Nascimento, X.P.R., Freitas-Silva, O., Soares, A.G., Costa, J.B.N., 2018. Compostos Organofosforados e seu Papel na Agricultura. Rev. Virtual Quim. 10, 172–193.

Benedetti, A.L., Vituri, C.L., Trentin, A.G., Domingues, M.A.C., Alvarez-Silva, M., 2004. The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb ®. Toxicol. Lett. 153, 227–232. https://doi.org/10.1016/j.toxlet.2004.04.008

Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A.B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K.E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R., Storer, T.W., 2001. Testosterone dose-response relationships in healthy young men. Am. J. Physiol. Endocrinol. Metab. 281, 1172–1181. https://doi.org/10.1152/ajpendo.2001.281.6.E1172

Blakemore, S., Burnett, S., Dahl, R.E., 2010. The Role of Puberty in the Developing Adolescent Brain. Hum. Brain Mapp. 31, 926–933. https://doi.org/10.1002/hbm.21052

Borck, P.C., Leite, N.C., Valcanaia, A.C., Rickli, S., Alípio, J.C.L., Machado, M., Vellosa, J.C., Mathias, P.C.F., Boschero, A.C., Grassiolli, S., 2020. Swimming training reduces glucose-amplifying pathway and cholinergic responses in islets from lean- and MSG-obese rats. Diabetes Obes Metab 47, 286–293. https://doi.org/10.1111/1440-1681.13197

Bright, J.E., Inns, R.H., Tuckwell, N.J., Griffiths, G.D., Marrs, T.C., 1991. A Histochemical Study of Changes Observed in the Mouse Diaphragm after Organophosphate Poisoning. Hum. Exp. Toxicol. 10, 9–14. https://doi.org/10.1177/096032719101000102

Camargo Filho, J.C.S., Vanderlei, L.C.M., Camargo, R.C.T., Oliveira, D.A.R., Oliveira Júnior, S.A., Dal Pai, V., Belangero, W.D., 2006. Análise histológica, histoquímica e morfométrica do músculo sóleo de ratos submetidos a treinamento físico em esteira rolante. Arq. Ciênc. Saúde 12, 196–199. https://doi.org/10.1590/S1517-86922011000600010

Cavalcanti, L.P.A.N., Aguiar, A.P., Lima, J.A., Lima, A.L.S., 2016. Intoxicação por organofosforados: tratamento e metodologias analíticas empregadas na avaliação da reativação e inibição da acetilcolinesterase. Rev. Virtual Quim. 8, 739–766.

Cavaliere, M.J., Calore, E.E., Perez, N.M., Puga, F.R., 1996. Miotoxicidade por organofosforados. Rev. Saúde Pública 30, 267–272. https://doi.org/10.1590/S0034-89101996000300010

Crabbs, T.A., 2021. NTP Nonneoplastic Lesion Atlas: Skeletal Muscle - Degeneration. [WWW Document]. URL https://ntp.niehs.nih.gov/nnl/musculoskeletal/skel_musc/degen/skeletal-muscle-degeneration_508.pdf (accessed 12.4.21).

De Bleecker, J.L., Abeele, K.G. van den, De Reuck, J.L., 1992. Variable involvement of rat skeletal muscles in paraoxon-induced necrotizing myopathy. Res. commun. chem. pathol. pharmacol. 75, 309–322.

Dutta, S., Sengupta, P., 2016. Men and mice: Relating their ages Sulagna. Life Sci. 152, 244–248. https://doi.org/10.1016/j.lfs.2015.10.025

Gallegos, C.E., Baier, C.J., Bartos, M., Bras, C., Domínguez, S., Mónaco, N., Gumilar, F., Giménez, M.S., Minetti, A., 2018. Perinatal Glyphosate-Based Herbicide Exposure in Rats Alters Brain Antioxidant Status, Glutamate and Acetylcholine Metabolism and Affects Recognition Memory. Neurotox Res. 34, 363–374. https://doi.org/10.1007/s12640-018-9894-2

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M., Séralini, G., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicol. 262, 184–191. https://doi.org/10.1016/j.tox.2009.06.006

Gaspari, R.J., Paydarfar, D., 2012. Respiratory recovery following organophosphate poisoning in a rat model is suppressed by isolated hypoxia at the point of apnea. Toxicology. 302, 242–247. https://doi.org/10.1016/j.tox.2012.08.005

Gill, J.P.K., Sethi, N., Mohan, A., Datta, S., Girdhar, M., 2018. Glyphosate toxicity for animals. Environ. Chem. Lett. 16, 401–426. https://doi.org/https://doi.org/10.1007/s10311-017-0689-0

Glusczak, L., Loro, V.L., Pretto, A., Moraes, B.S., Raabe, A., Duarte, M.F., Fonseca, M.B., Menezes, C.C., Valladão, D.M.S., 2011. Acute Exposure to Glyphosate Herbicide Affects Oxidative Parameters in Piava (Leporinus obtusidens). Arch. Environ. Contam. Toxicol. 61, 624–630. https://doi.org/10.1007/s00244-011-9652-4

Gomez, A.L., Altamirano, G.A., Tschopp, M. V., Bosquiazzo, V.L., Muñoz-de-toro, M., Kass, L., 2020. Exposure to a Glyphosate-based Herbicide Alters the Expression of Key Regulators of Mammary Gland Development on Pre-pubertal Male Rats. Toxicol. 439, 152477. https://doi.org/10.1016/j.tox.2020.152477

Greaves, P., Chouinard, L., Ernst, H., Mecklenburg, L., Pruimboom-brees, I.M., Rinke, M., Rittinghausen, S., Thibault, S., von Erichsen, J., Yoshida, T., 2013. Proliferative and Non-Proliferative Lesions of the Rat and Mouse. J. Toxicol. Pathol. 26, 1S–26S. https://doi.org/10.1293/tox.26.1S

Greenspan, L.C., Lee, M.M., 2019. Endocrine Disrupters and Pubertal Timing Louise. Endocrinol Diabetes Obes. 25, 49–54. https://doi.org/10.1097/MED.

Herbst, K.L., Bhasin, S., 2004. Testosterone action on skeletal muscle. Curr. Opin. Clin. Nutr. Metab. Care. 7, 271–277. https://doi.org/10.1097/01.mco.0000126345.96117.9c

Hoppin, J.A., Umbach, D.M., Long, S., Rinsky, J.L., Henneberger, P.K., Salo, P.M., Zeldin, D.C., London, S.J., Alavanja, M.C.R., Blair, A., Freeman, L.E.B., Sandler, D.P., 2014. Respiratory disease in United States farmers. J. Occup. Environ. Med. 71, 484–491. https://doi.org/10.1136/oemed-2013-101983

Jasper, R., Locatelli, G.O., Pilati, C., Locatelli, C., 2012. Evaluation of biochemical, hematological and oxidative parameters in mice exposed to the herbicide glyphosate-Roundup®. Interdiscip. Toxicol. 5, 133–140. https://doi.org/10.2478/v10102-012-0022-5

Junqueira, L.C.U., Bignolas, G., Brentani, R.R., 1979. Picrosirius staining plus polarization microscopy , a specific method for collagen detection in tissue sections. Histochem. J. 11, 447–455. https://doi.org/ 10.1007/BF01002772

Junqueira, L.C.U., Junqueira, L.M.M.S., 1983. Técnicas básicas de citologia e histologia. São Paulo: Santos Editora.

Kabitz, H., Walker, D., Schwoerer, A., Sonntag, F., Walterspacher, S., Roecker, K., Windisch, W., 2007. New physiological insights into exercise-induced diaphragmatic fatigue. Respir. Physiol. Neurobiol. 158, 88–96. https://doi.org/10.1016/j.resp.2007.04.011

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. 27, 1A-149A.

Kjaer, M., Magnusson, P., Krogsgaard, M., Møller, J.B., Olesen, J., Heinemeier, K., Hansen, M., Haraldsson, B., Koskinen, S., Esmarck, B., Langberg, H., 2006. Extracellular matrix adaptation of tendon and skeletal muscle to exercise. J. Anat. 208, 445–450. https://doi.org/10.1111/j.1469-7580.2006.00549.x

Klein-rodewald, T., Seeger, T., Dutschmann, M., Worek, F., Mörschel, M., 2011. Central respiratory effects on motor nerve activities after organophosphate exposure in a working heart brainstem preparation of the rat. Toxicol. Lett. 206, 94–99. https://doi.org/10.1016/j.toxlet.2011.07.005

Lajmanovich, R.C., Attademo, A.M., Peltzer, P.M., Junges, C.M., Cabagna, M.C., 2011. Toxicity of Four Herbicide Formulations with Glyphosate on Rhinella arenarum (Anura: Bufonidae) Tadpoles: B-esterases and Glutathione S-transferase Inhibitors. Arch. Environ. Contam. Toxicol. 60, 681–689. https://doi.org/10.1007/s00244-010-9578-2

Lehrer, G.M., Ornstein, L., 1959. A diazo coupling method for the electron microscopic localization of cholinesterase. J. Biophys. Biochem. Cytol. 6, 399–419. https://doi.org/10.1083/jcb.6.3.399.

Lucaccioni, L., Trevisani, V., Marrozzini, L., Bertoncelli, N., Predieri, B., Lugli, L., Berardi, A., Iughetti, L., 2020. Endocrine-Disrupting Chemicals and Their Effects during Female Puberty: A Review of Current Evidence. Int. J. Mol. Sci. 21, 2078. https://doi.org/10.3390/ijms21062078

Mesnage, R., Arno, M., Costanzo, M., Malatesta, M., Séralini, G., Antoniou, M.N., 2015. Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. Environ. Heal. 14, 70. https://doi.org/10.1186/s12940-015-0056-1

Milesi, M.M., Lorenz, V., Durando, M., Rossetti, M.F., Varayoud, J., 2021. Glyphosate Herbicide: Reproductive Outcomes and Multigenerational Effects. Front. Endocrinol. 12, 672532. https://doi.org/10.3389/fendo.2021.672532

Moon, J., Chun, B., Lee, S., 2015. Variable response of cholinesterase activities following human exposure to different types of organophosphates. Hum. Exp. Toxicol. 34, 698–706. https://doi.org/10.1177/0960327114558890

Moura, F.R., Brentegani, K.R., Gemelli, A., Sinhorin, A.P., Sinhorin, V.D.G., 2017. Oxidative stress in the hybrid fish jundiara (Leiarius marmoratus x Pseudoplatystoma reticulatum) exposed to Roundup Original®. Chemosphere. 185, 445–451. https://doi.org/10.1016/j.chemosphere.2017.07.030

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W. V, Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. J. Environ. Heal. 15, 19. https://doi.org/10.1186/s12940-016-0117-0

Nardi, J., Moras, P.B., Koeppe, C., Dallegrave, E., Leal, M.B., Rossato-Grando, L.G., 2017. Prepubertal subchronic exposure to soy milk and glyphosate leads to endocrine disruption. Food Chem. Toxicol. 100, 247–252. https://doi.org/10.1016/j.fct.2016.12.030

Neves, P.D.M., Bellini, M., 2013. Intoxicações por agrotóxicos na mesorregião norte central paranaense, Brasil – 2002 a 2011. Ciênc. Saúde Colet. 18, 3147–3156. https://doi.org/10.1590/S1413-81232013001100005

Orozco-Levi, M., Servei, J.G., 1997. El diafragma. Arch. Bronconeumol. 33, 399–411. https://doi.org/10.1016/S0300-2896(15)30567-6

Osuna-Flores, I., Pérez-Morales, A., Olivos-Ortiz, A., Álvarez-González, C.A., 2019. Effect of organophosphorus pesticides in juveniles of Litopenaeus vannamei: alteration of glycogen, triglycerides, and proteins. Ecotoxicology.

Owagboriaye, F., Dedeke, G., Ademolu, K., Olujimi, O., Aladesida, A., Adeleke, M., 2019. Comparative studies on endogenic stress hormones, antioxidant, biochemical and hematological status of metabolic disturbance in albino rat exposed to roundup herbicide and its active ingredient glyphosate. Environ. Sci. Pollut. Res. 26, 14502–14512. https://doi.org/10.1007/s11356-019-04759-1

Pandey, A., Dhabade, P., Kumarasamy, A., 2019. Inflammatory Effects of Subacute Exposure of Roundup in Rat Liver and Adipose Tissue. Dose-Response. 17, 1–11. https://doi.org/10.1177/1559325819843380

Pandey, A., Rudraiah, M., 2015. Analysis of endocrine disruption effect of Roundup ® in adrenal gland of male rats. Toxicol. Rep. 2, 1075–1085. https://doi.org/10.1016/j.toxrep.2015.07.021

Pervanidou, P., Chrousos, G.P., 2012. Metabolic consequences of stress during childhood and adolescence. Metabolism. 61, 611–619. https://doi.org/10.1016/j.metabol.2011.10.005

Pham, T.H., Derian, L., Kervarrec, C., Kernanec, P., Jegou, B., Smagulova, F., Gély-pernot, A., 2019. Perinatal exposure to glyphosate and a glyphosate-based herbicide affect spermatogenesis in mice. Toxicol. Sci. 169, 260–271. https://doi.org/10.1093/toxsci/kfz039

Possamai, F.P., Fortunato, J.J., Feier, G., Agostinho, F.R., Quevedo, J., Filho, D.W., Dal-pizzol, F., 2007. Oxidative stress after acute and sub-chronic malathion intoxication in Wistar rats. Environ. toxicol. pharmacol. 23, 198–204. https://doi.org/10.1016/j.etap.2006.09.003

Prakash, Y.S., Zhan, W.Z., Miyata, H., Sieck, G.C., 1995. Adaptations of Diaphragm Neuromuscular Junction following Inactivity. Acta anat. 154, 147–161. https://doi.org/10.1159/000147762

Rathinam, X., Kota, R., Thiyagar, N., 2005. Farmers and Formulations – Rural Health Perspective. Malays. 60, 118–125.

Ren, X., Li, R., Liu, J., Huang, K., Wu, S., Li, Y., Li, C., 2018. Effects of glyphosate on the ovarian function of pregnant mice, the secretion of hormones and the sex ratio of their fetuses. Environ. Pollut. 243, 833–841. https://doi.org/10.1016/j.envpol.2018.09.049

Reynoso, E.C., Torres, E., Bettazzi, F., Palchetti, I., 2019. Trends and Perspectives in Immunosensors for Determination of Currently-Used Pesticides: The Case of Glyphosate, Organophosphates, and Neonicotinoids. Biosensors. 9, 20. https://doi.org/10.3390/bios9010020

Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., Souza, P.B., Oliveira, P.V., Bernardi, M.M., Nunes, M.T., Oliveira, C.A., 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch Toxicol 86, 663–673. https://doi.org/10.1007/s00204-011-0788-9

Romano, R.M., Romano, M.A., Oliveira, C.A., 2009. Glifosato como desregulador endócrino químico. Ambiência - Rev. do Set. Ciências Agrárias e Ambient. 5, 359–372.

Ros, L.F., Teodozio, C.P., Julia, A., Costa, A., Rossi, R.C., Batista, R., Pacagnelli, F.L., 2020. Análise dos efeitos da exposição crônica oral ao herbicida à base de glifosato na remodelação cardíaca em ratos. Vitae. 12, 10–15.

Rowley, K.L., Mantilla, C.B., Sieck, G.C., 2005. Respiratory muscle plasticity. Respir Physiol Neurobiol 147, 235–251. https://doi.org/10.1016/j.resp.2005.03.003

Santos, V.C., Vechetti Júnior, I.J., Sant'Ana, D.M.G., Almeida, F.L.A., 2019. Vias de sinalizaçãocelular envolvidas no crescimento e manutenção do músculo estriado esquelético. Arq Mudi. 23, 220–248. https://doi.org/10.4025/arqmudi.v23i3.51539

Selmi, S., Rtibi, K., Grami, D., Sebai, H., Marzouki, L., 2018. Malathion, an organophosphate insecticide, provokes metabolic, histopathologic and molecular disorders in liver and kidney in prepubertal male mice. Toxicol. Rep. 5, 189–195. https://doi.org/10.1016/j.toxrep.2017.12.021

Sieck, D.C., Zhan, W., Fang, Y., Ermilov, L.G., Sieck, G.C., Mantilla, C.B., 2012. Structure–activity relationships in rodent diaphragm muscle fibers vs. neuromuscular junctions. Respir. Physiol. Neurobiol. 180, 88–96. https://doi.org/10.1016/j.resp.2011.10.015

Sihvo, H.K., Immonen, K., Puolanne, E., 2013. Myodegeneration With Fibrosis and Regeneration in the Pectoralis Major Muscle of Broilers. Vet. Pathol 00, 1–5. https://doi.org/10.1177/0300985813497488

Silva, F.L., Mazzotti, N.G., Picoral, M., Nascimento, D.M., Isabel, M., Martins, M., Klein, A.B., 2005. Infarto do miocárdio experimental e aumento do estresse oxidativo em diafragma de ratos*. J. Bras. Pneumol. 31, 506–510.

Silva, K.N., Martins, N.C., Silveira, J.M., Reis, G.R., 2012. Músculos respiratórios: fisiologia, avaliação e protocolos de treinamento. Rev. Cereus. 6, 1–13.

Silva, M.R., Campos, A.C.E., Bohm, F.Z., 2013. Agrotóxicos e seus impactos sobre ecossistemas aquáticos continentais. Rev. Saúde e Biol. 8, 46–58.

Slager, R.E., Simpson, S.L., Levan, T.D., Poole, J.A., Sandler, D.P., Hoppin, J.A., 2010. Rhinitis Associated with Pesticide Use Among Private Pesticide Applicators in the Agricultural Health Study. J. toxicol. environ. Heal. 73, 1382–1393. https://doi.org/10.1080/15287394.2010.497443.

Soares, W., Almeida, R.M.V.R., Moro, S., 2003. Trabalho rural e fatores de risco associados ao regime de uso de agrotóxicos em Minas Gerais, Brasil. Cad. Saúde Pública. 19, 1117–1127. https://doi.org/10.1590/S0102-311X2003000400033

Souza, J.S., Kizys, M.M.L., Conceição, R.R., Glebocki, G., Romano, R.M., Ortiga-Carvalho, T.M., Giannocco, G., Silva, I.D.C.G., Dias da Silva, M.R., Romano, M.A., Chiamolera, M.I., 2017. Perinatal exposure to glyphosate-based herbicide alters the thyrotrophic axis and causes thyroid hormone homeostasis imbalance in male rats. Toxicology 377, 25–37. https://doi.org/10.1016/j.tox.2016.11.005

Souza, T.O.F., Mesquita, D.A., Ferrari, R.A.M., Pinto Jr, D.S., Correa, L., Bussadori, S.K., Fernandes, K.P.S., Martins, M.D., 2011. Phototherapy with low-level laser affects the remodeling of types I and III collagen in skeletal muscle repair. Lasers Med. Sci. 26, 803–814. https://doi.org/10.1007/s10103-011-0951-9

Tang, J., Hu, P., Li, Y., Win-Shwe, T.T., Li, C., 2017. Ion Imbalance Is Involved in the Mechanisms of Liver Oxidative Damage in Rats Exposed to Glyphosate. Front. physiol. 8, 1–12. https://doi.org/10.3389/fphys.2017.01083

Teixeira, J.M. dos S., Lima, V. da S., Moura, F.R., Marisco, P. da C., Sinhorin, A.P., Sinhorin, A.P., 2018. Acute toxicity and effects of Roundup Original® on pintado da Amazônia. Environ. Sci. Pollut. Res. Int. 25, 25383–25389. https://doi.org/10.1007/s11356-018-2630-x

Turkmen, R., Birdane, Y.O., Demirel, H.H., Kabu, M., Ince, S., 2019. Protective effects of resveratrol on biomarkers of oxidative stress, biochemical and histopathological changes induced by sub-chronic oral glyphosate- based herbicide in rats. Toxicol. Res. 8, 238–245.

Van der Meer, S.F.T., Jaspers, R.T., Degens, H., 2011. Is the myonuclear domain size fixed? J. musculoskelet. interact. 11, 286–297.

WHO/UNEP, 2013. State of the science of endocrine-disrupting chemicals - 2012., in: Bergman, A., Heindel, J.J., Jobling, S., Kidd, A.K., Zoeller, R.T. (Eds.), . United Nations Environment Programme and the World Health Organization, Geneva, p. 296.

Apendicês

Tabela 1. Parâmetros macroscópicos de camundongos com 150 dias de idade dos grupos controle (CTL) e herbicida à base de glifosato (GBH).

	CTL	GBH
Peso corporal (g)[a]	30,25 ± 1,70	27,75 ± 2,33*
Comprimento nasoanal (cm) [a]	9,11 ± 0,43	8,95 ± 0,18
Gordura retroperitoneal (% PC) [b]	0,28 ± 0,11	0,34 ± 0,19
Gordura perigonadal (% PC) [a]	1,12 ± 0,33	1,25 ± 0,49
Peso do músculo diafragma (g) [a]	0,04 ± 0,01	0,04 ± 0,00

Valores expressos como média ± desvio padrão. [a] Teste t de Student. [b] Teste MannWhitney. *representa diferença significativa (p< 0,05).

Tabela 2. Avaliação morfométrica das fibras musculares do músculo diafragma de camundongos com 150 dias de idade, dos grupos controle (CTL) e herbicida à base de glifosato (GBH).

	CTL	GBH
Área (μm²)	742,38 ± 96,78	841,35 ± 88,48*
Diâmetro maior (μm)	36,70 ± 2,42	39,95 ± 1,82*
Diâmetro menor (μm)	23,64 ± 1,61	25,13 ± 1,67*
Densidade de fibras	994,40 ± 180,06	913,40 ± 66,21
Número de núcleos periféricos	1322,60 ± 210,21	1242,60 ± 77,61
Número de núcleos centrais	42,20 ± 4,21	30,40 ± 15,31
Relação de núcleo/fibra	1,35 ± 0,27	1,37 ± 0,13
Número de capilares	489,60 ± 56,05	469,00 ± 49,31
Relação de capilar/fibra	0,50 ± 0,10	0,52 ± 0,09

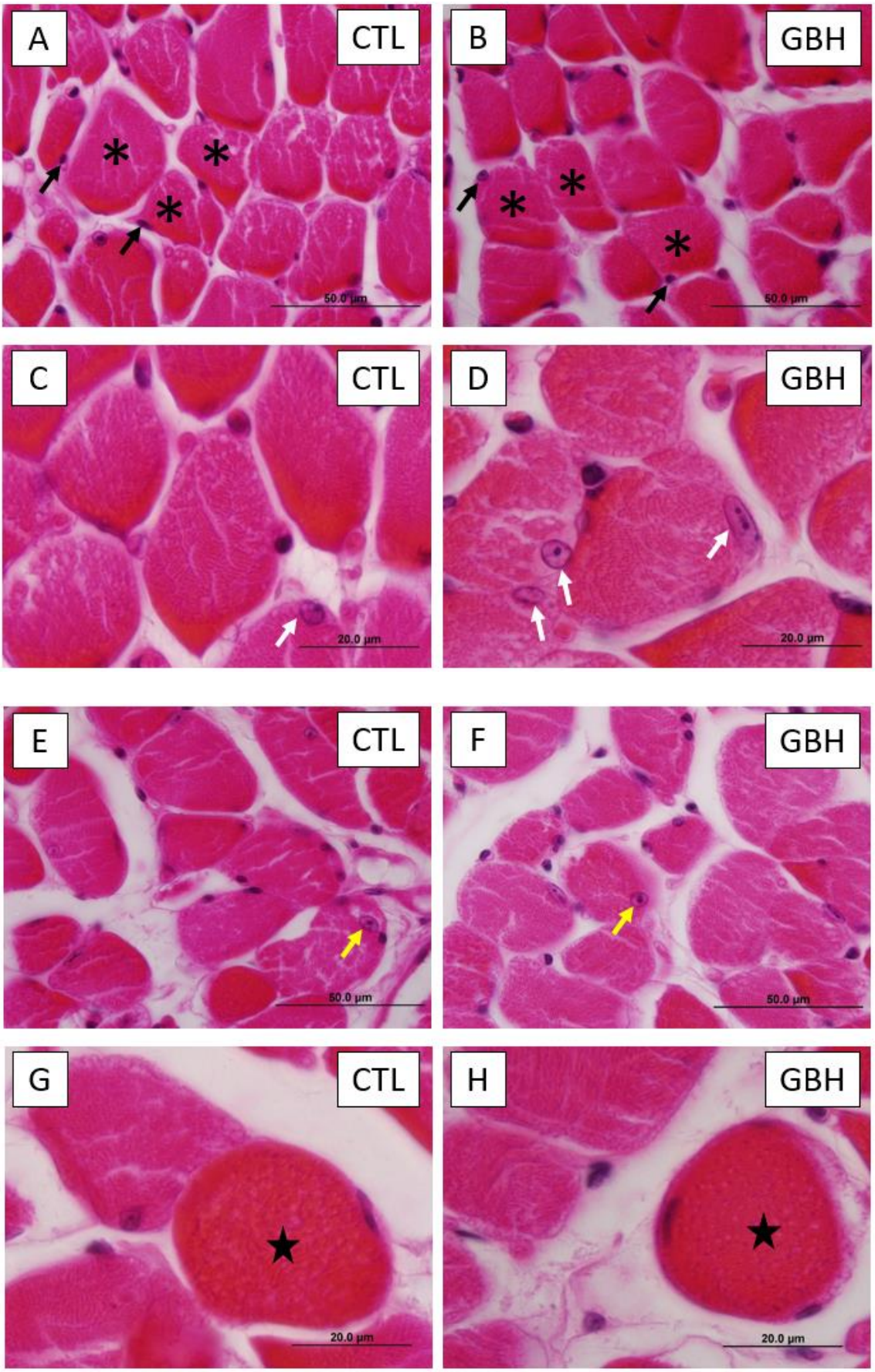
Valores expressos como média ± desvio padrão. Teste *t* de Student. *representa diferença significativa (p< 0,05).

Tabela 3. Avaliação quantitativa do tecido conjuntivo do músculo diafragma de camundongos com 150 dias de idade, dos grupos controle (CTL) e herbicida à base de glifosato (GBH).

	CTL	GBH	P-valor
Estimativa do tecido conjuntivo (%)	2,49 ± 0,83	1,86 ± 0,46	0,1058
Colágeno tipo I	17,17 ± 5,16	17,24 ± 4,28	0,9802

Colágeno tipo III	29,31 ± 7,53	40,04 ± 8,10	0,0205*
Sobreposição do colágeno tipo I e III	53,52 ± 5,54	42,72 ± 10,85	0,0097*

Valores expressos como média ± desvio padrão. Teste *t* de Student. *representa diferença significativa (p< 0,05).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CTL
50.0 µm
B
GBH
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C
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D
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E
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F
GBH
50.0 µm
G
CTL
20.0 µm
H
GBH
20.0 µm

Figura 1. Fotomicrografias do músculo diafragma de camundongos com 150 dias de idade. Secção transversal, HE. **A** e **B:** Fibras músculares (asterisco) e núcleos periféricos (setas pretas). **C** e **D**: Núcleos basofílicos aumentados (setas brancas). **E** e **F**: Núcleos centralizados (setas amarela). **G** e **H:** Fibras musculares arredondadas com diferença tintorial (estrela). CTL: Grupo controle. GBH: Grupo herbicida à base de glifosato.

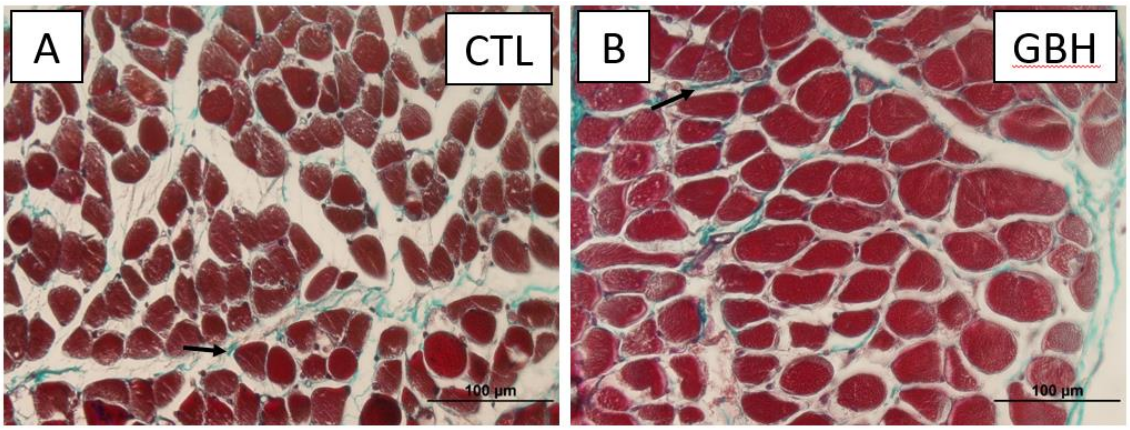

Figura 2. Fotomicrografias do músculo diafragma de camundongos com 150 dias de idade. Secção transversal, Tricrômico de Masson. **A e B:** Observar o perímisio (seta). CTL: Grupo controle. GBH: Grupo herbicida à base de glifosato.

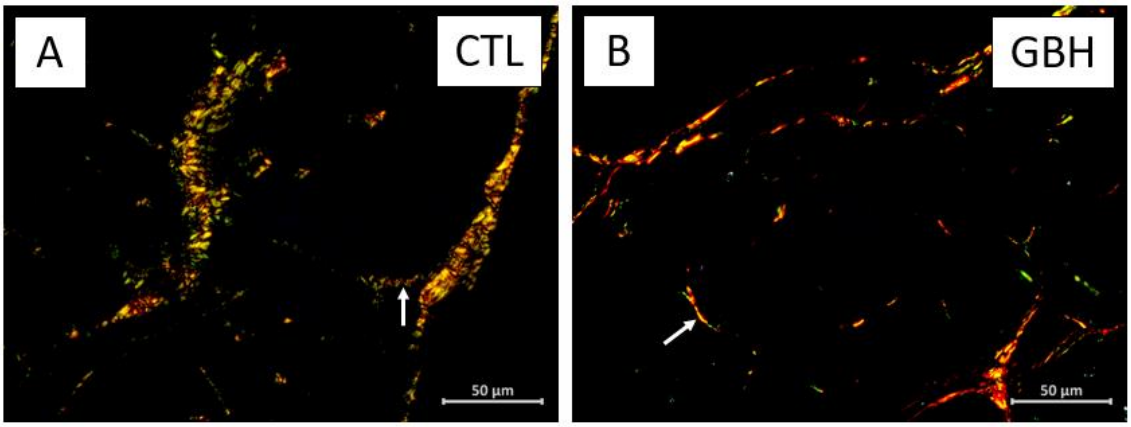

Figura 3. Fotomicrografias do músculo diafragma de camundongos com 150 dias de idade. Secção transversal, Picrossirius Red. **A e B:** Observar o perímisio (seta). Colágeno do tipo I (em verde), colágeno do tipo III (em vermelho) e áreas de sobreposição de tipo colágeno I e III (em amarelo). CTL: Grupo controle. GBH: Grupo herbicida à base de glifosato.

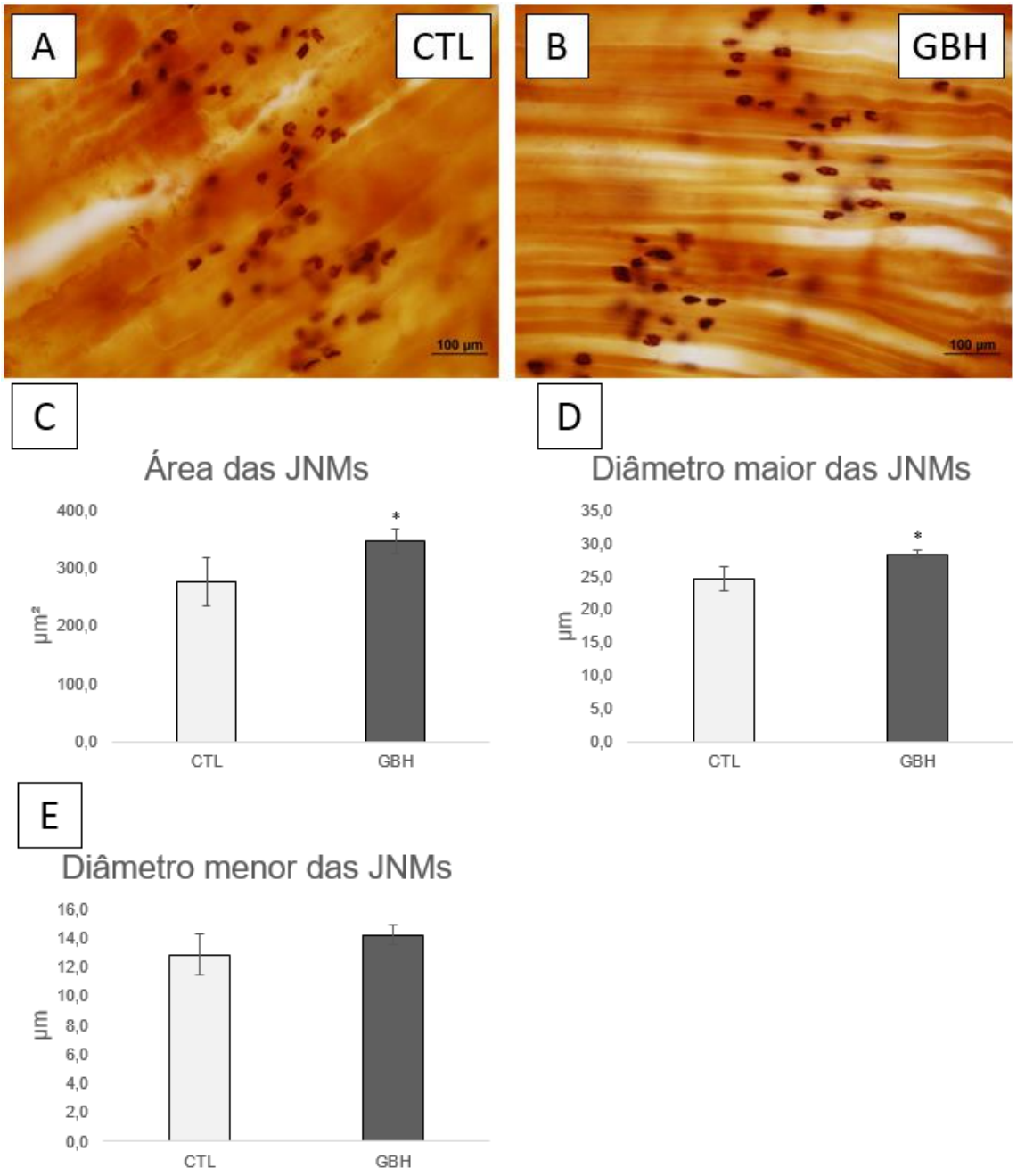

Figura 4. Fotomicrografias das junções neuromusculares (JNMs) observadas no músculo diafragma de camundongos com 150 dias de idade. Secção longitudinal, reação de Esterase Inespecífica. **A e B:** Características morfológicas das JNMs. **C, D e E:** Dados de área, diâmetro maior e diâmetro menor das JNMs. CTL: Grupo controle. GBH: Grupo herbicida à base de glifosato. Valores expressos como média ± desvio padrão. *representa diferença significativa ($p < 0,05$). Teste *t* de Student aplicado nos dados da área e diâmetro maior das JNMs. Teste Mann-Whitney foi realizado nos dados do diâmetro menor das JNMs.

ARTIGO CIENTÍFICO 2

PERIPUBERTAL EXPOSURE TO GLYPHOSATE-BASED HERBICIDE MODIFIES THE EFFECTS OF A HIGH-FAT DIET ON DIAPHRAGM HISTOMORPHOMETRY OF MICE

REVISTA MICRON

NORMAS DA REVISTA: ANEXO 03

Peripubertal exposure to glyphosate-based herbicide modifies the effects of a high-fat diet on diaphragm histomorphometry of mice

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Abstract

According to the theory of the developmental origins of health and disease, exposure to the pesticide glyphosate during critical periods of development may be related to obesity in adulthood. Obesity is characterized by a high level of body fat that compromises the morphophysiology of several organ systems, including the musculoskeletal system. The aim of this study was to determine whether peripubertal exposure to glyphosate-based herbicide influences the deleterious effects of obesity induced by a high-fat diet on the diaphragm muscle of adult mice. Ten male C57BL/6 mice were divided into two groups (n = 5 per group): a control group that received a high-fat diet and water by gavage, and an experimental group that received a high-fat diet and glyphosate-based herbicide (50 mg Roundup®/kg per day by gavage) from 30 to 60 days of life. From 60 to 150 days, both groups received the high-fat diet. At 150 days, the animals were euthanized and weighed. The nasoanal length was measured and fats and diaphragm were collected and weighed. The muscle was stored in specific fixatives for histological analysis of muscle fibers and neuromuscular junctions (NMJs). There was no difference in macroscopic parameters between groups. Morphological analysis of muscle fibers showed degenerative changes in the diaphragm of both groups; however, these alterations were more marked in the group exposed to the glyphosate-based herbicide. The area and maximum diameter of NMJs were also greater in the herbicide-exposed group. In conclusion, obesity promotes degenerative changes in diaphragm muscle fibers of adult mice. Peripubertal exposure to glyphosate-based herbicide exacerbates the myotoxic effects of obesity and increases the area and maximum diameter of NMJs in obese adult mice.

Keywords: Pesticides; Glyphosate; Toxicity; Obesity; Skeletal Muscle Fibers; Diaphragm

1. Introduction

Developmental Origins of Health and Disease (DOHaD) is an area of science that investigates the effects caused by insults that occur at critical stages of development and that can result in physiological, morphological and metabolic changes, consequently affecting health and disease patterns throughout life (Gluckman et al., 2010; Marques et al., 2021; Suzuki, 2017). The different environmental insults that harm human health include the effects of pesticides (Suzuki, 2017). Pesticides and many other industrial chemicals, plasticizers, pharmaceuticals, and metals are considered endocrine disruptors (Kalofiri et al., 2021; Lucaccioni et al., 2020; WHO/UNEP, 2013; Yilmaz et al., 2020). Ingested through contaminated food, water or air or through skin contact (Kalofiri et al., 2021; Schneider et al., 2019), these substances or exogenous mixtures can compromise normal endocrine function (WHO/UNEP, 2013; Yilmaz et al., 2020) and can lead to the development of a variety of reproductive, neurological or metabolic pathologies in the body (Schneider et al., 2019), offspring or subpopulations (WHO/UNEP, 2013).

Puberty is currently recognized as an important phase of life since it is characterized by a high degree of plasticity (Cerf et al., 2015; Ho et al., 2016; Silveira et al., 2007), and is therefore susceptible to the effects of endocrine disruptors whose deleterious effects may only manifest in adulthood (Boudalia et al., 2021; Kalofiri et al., 2021; Newbold et al., 2008). Hence, endocrine changes induced by chemical compounds during this sensitive period can lead to deleterious effects in adulthood, supporting the DOHaD theory (Ivanski et al., 2020).

Glyphosate [N-(phosphonomethyl)glycine], an organophosphate herbicide (Barbosa et al., 2021), is the most widely used herbicide in the world and the active ingredient of all glyphosate-based herbicides (Milesi et al., 2021), including Roundup® (Owagboriaye et al., 2019). Its herbicidal activity is exacerbated when combined with other chemical products, called co-formulants (Milesi et al., 2021). Glyphosate kills weeds and grasses by inhibiting 5-enolpyruvate-shikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimic acid metabolic pathway. Since this pathway does not occur in vertebrates, glyphosate is considered to be safe for mammals (Ingaramo et al., 2020; Mesnage et al., 2015; Romano et al., 2009). However, evidence accumulated over time has demonstrated the harmful effects of glyphosate-based herbicides on different species (George et al., 2010; Peixoto, 2005; Schimpf et al., 2017; Walsh et al., 2000), including humans (Bai and Ogbourne, 2016; Benachour et

al., 2007; Richard et al., 2005; Thakur et al., 2014). These herbicides are considered potential endocrine disruptors (Richard et al., 2005; Romano et al., 2009; Teleken et al., 2020).

Exposure to glyphosate can cause oxidative stress, affecting the nervous system, reproductive system and organs such as liver and kidneys (Soudani et al., 2019), as well as changes in the endocrine system (Owagboriaye et al., 2019). The high rate of morbidity observed in organophosphate poisoning is associated with the involvement of skeletal striated muscles, especially respiratory muscles (Cavaliere et al., 1996).

Evidence indicates that exposure of humans to endocrine-disrupting chemicals at critical stages of development may also be linked to obesity (Baillie-Hamilton et al., 2002; Boudalia et al., 2021; Newbold et al., 2008), a disease characterized by excess body fat caused by the complex interaction between genetic and environmental factors (especially eating habits and sedentarism). Obesity has reached global epidemic proportions, a fact that renders it a human health crisis (Boudalia et al., 2021; Newbold et al., 2008), causing or aggravating many health problems in children, adolescents, and adults (Li et al., 2015). The short-term effects of obesity include cardiometabolic, respiratory, musculoskeletal, endocrine and psychosocial consequences and an increase in cancer risk (Newbold et al., 2008); in addition, the disease affects and accompanies a large proportion of children and adolescents into adulthood, causing different chronic diseases (Petrovics et al., 2021).

The lower percentage of muscle mass in obese individuals is associated with diseases such as hypertension and diabetes mellitus (Sizoo et al., 2021). Obesity can also affect respiratory function, including the diaphragm muscle, as a result of greater respiratory effort and impaired gas exchange. In addition, abdominal muscle hypertonia caused by obesity can compromise respiratory function dependent on diaphragmatic action as a result of reduced muscle performance and chest expansion (Rasslan et al., 2009). A recent study has demonstrated an inverse association between obesity and cardiorespiratory and muscle performance in adolescents. Overweight was also inversely correlated with cardiorespiratory performance during the 4-year follow-up period (Petrovics et al., 2021).

It is therefore important to identify obesogenic substances and the possible correlation of pesticides such as glyphosate with the development of obesity (Newbold et al., 2008). The aim of this study was to determine whether peripubertal exposure to a glyphosate-based herbicide (GBH) influences the deleterious effects of obesity induced by a high-fat diet (HFD) on the diaphragm muscle of adult mice.

2. Materials and methods

2.1 Ethical aspects

The Ethics Committee on Animal Use of Universidade Estadual do Oeste do Paraná (UNIOESTE) approved all experimental procedures on May 11, 2018. The institutional guidelines for the use of animals in research were followed for maintenance and care of animals.

2.2 Experimental procedures

2.2.1 Animals

Ten male C57BL/6 mice, 21 days old and mean body weight of 20 g, were used. The animals were obtained from the central animal house of UNIOESTE and were kept in the sectorial animal house of the Laboratory of Endocrine Physiology and Metabolism (LAFEM) under controlled conditions of temperature (28 ± 2ºC) and lighting (12-h light/dark).

2.2.2 Experimental groups

At 30 days of age, the animals were weaned and randomly divided into two groups. Only two offspring of the same dam were allocated to the same group: control group (CTL, n=5) and glyphosate-based herbicide group (GBH, n=5). From 30 to 60 days of age, the CTL group received water and the GBH group received 50 mg/kg body weight (Romano et al., 2008) of Roundup® Original DI (Monsanto, Brazil), which contained 445 g/L of N-(phosphonomethyl)glycine diammonium salt, corresponding to 370 g/L or 37.0% (w/v) of the active component glyphosate. Both groups were fed daily the established solution by gavage, always at the same time (Figure 1). The pH of the GBH solution and water was corrected to 5-5.5 with hydrochloric acid. All mice had *ad libitum* access to water and were fed a standard rodent diet (Nuvital, Brazil) during this period.

2.2.3 Induction of obesity

Once the animals had completed 60 days of life, the CTL and GBH groups were exposed to the HFD as follows: high-fat diet control (HFD-CTL group, n=5) – animals received water by gavage and were fed the standard diet from 30 to 60 days and the HFD from 60 to 150 days for the induction of obesity; high-fat diet + glyphosate-based herbicide (HFD-GBH group, n=5) – GBH was administered by gavage and the animals were fed the standard diet from 30 to 60 days and the HFD from 60 to 150 days (Figure 1).

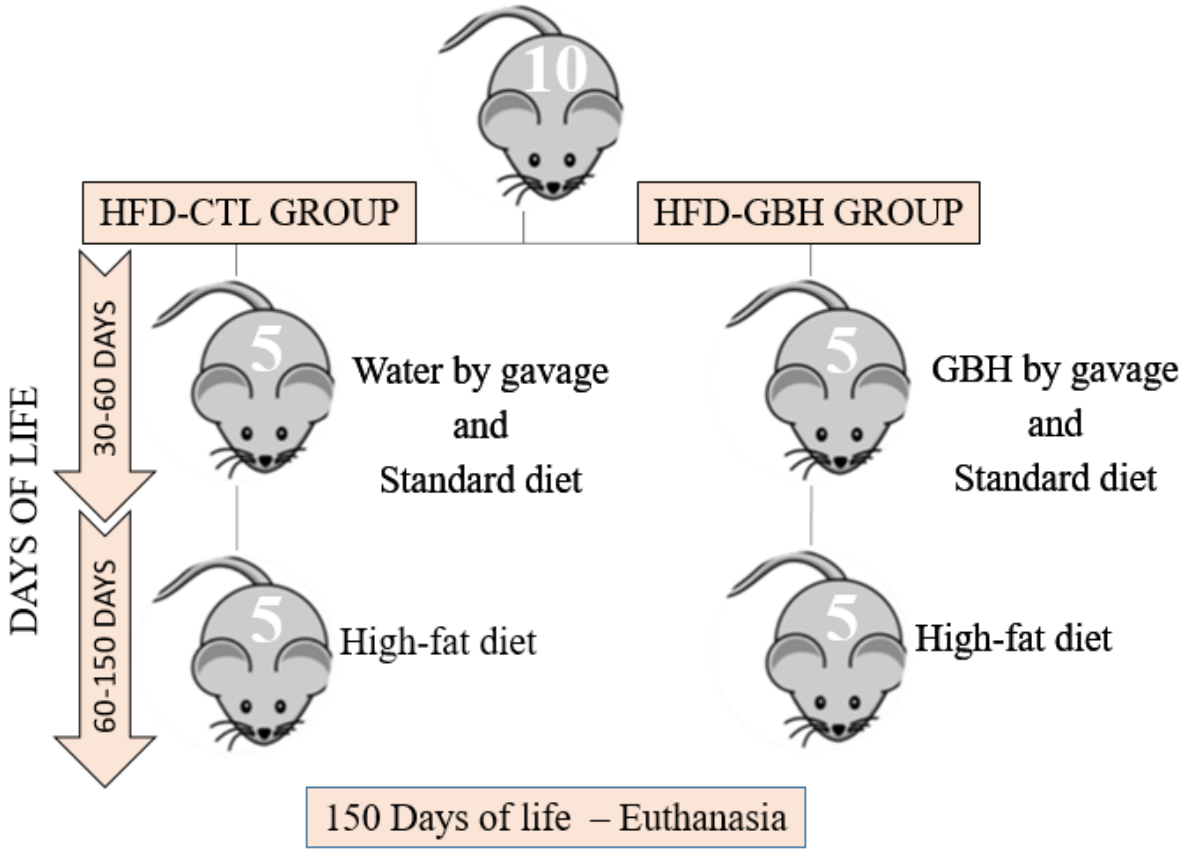

Figure 1. Flow diagram of the experimental design. HFD-CTL: high-fat diet control. HFD-GBH: high-fat diet + glyphosate-based herbicide. GBH: glyphosate-based herbicide. Source: The authors.

The standard diet for laboratory rodents consisted of 70% carbohydrates, 20% proteins and 10% fats, containing 3.8 kcal/g. The diet was obtained from the central animal house of UNIOESTE (Nuvital, Brazil). The HFD was prepared with 50% triturated standard ration, 31.2% pork fat, 14.8% casein, and 4% soybean oil, containing 6.2 kcal/g.

2.2.4 Euthanasia and tissue collection

At 150 days of age, the animals were weighed and the nasoanal length was measured. These measurements were used for calculation of the Lee index [body weight (g) 1/3 / nasoanal length (cm) × 1000] (Bernardis and Patterson, 1968; Fernandes et al., 2012). The animals were anesthetized with a mixture of xylazine hydrochloride (9 mg/kg; Anasedan®, Vetbrands, Brazil) and ketamine hydrochloride (90 mg/kg; Dopalen®, Vetbrands, Brazil). Laparotomy was then performed. The retroperitoneal and perigonadal fat was collected and weighed. For collection of the diaphragm muscle, the animals were placed in a ventral position and a midline incision was made just below the thorax, followed by elevation of the skin and muscles. Only the costal part of the diaphragm was removed and divided into right and left antimeres. Each antimere was cut into two fragments. The upper and lower fragments of the right antimere were processed and embedded in paraffin for the histological study of muscle fibers and the upper and lower fragments of the left antimere were submitted to histochemical staining for the study of neuromuscular junctions (NMJs).

2.2.5 Histological study of muscle fibers

The fragments of the right antimere of the diaphragm were fixed in methacarn, cut transversely, and stained with hematoxylin-eosin (Junqueira and Carneiro, 2013). The stained slides were used for the quantification of muscle fibers, peripheral and central nuclei, capillaries, nucleus/fiber ratio, and capillary/fiber ratio. Ten microscopic fields (40x objective) were analyzed per animal.

2.2.6 Morphological and morphometric study of NMJs

The fragments of the left antimere of the diaphragm were fixed in Karnovsky solution (Karnovsky, 1965) and cut longitudinally into several thin sections with a stainless-steel blade. The sections were submitted to the nonspecific esterase reaction (Lehrer and Ornstein, 1959). Morphometric analysis of NMJs consisted of the measurement of the area and maximum and minimum diameters of 100 NMJs per animal on microscopic images (20x objective).

2.3 Image analysis

The images of muscle fibers and NMJs were acquired with an Olympus Bx60® microscope coupled to an Olympus DP71 camera (Tokyo, Japan) using the DP Controler 3.2.1 276 software. The Image Pro Plus 6.0® program (Media Cybernetics, Maryland, USA) was used for the morphometric analysis of muscle fibers and NMJs.

2.4 Statistical analysis

The data were analyzed statistically using the GraphPad Prism® program (La Jolla, USA). Normality was evaluated by the Kolmogorov-Smirnov test. The Student *t*-test was applied and a p value < 0.05 was considered to be statistically significant.

3. Results

3.1 Macroscopic parameters

There was no significant difference in macroscopic parameters between the HFD-GBH and HFD-CTL groups (Table 1).

Table 1. Macroscopic parameters of C57BL/6 mice at 150 days of age in the high-fat diet control group and high-fat diet + glyphosate-based herbicide group.

Macroscopic parameters	Groups		P value
	HFD-CTL	**HFD-GBH**	
Body weight (g)	39.73±4.02	38.06±4.73	0.5274
Nasoanal length (cm)	9.28±0.22	9.54±0.26	0.1216
Lee index	367.46±11.44	352.16±14.52	0.2351
Retroperitoneal fat (g)	0.69±0.07	0.67±0.12	0.7768
Perigonadal fat (g)	2.13±0.38	1.83±0.49	0.4439
Diaphragm muscle weight (mg)	0.05±0	0.04±0	0.2875

Values are expressed as the mean ± standard deviation. Kolmogorov-Smirnov test. Student *t*-test ($p < 0.05$). HFD-CTL: high-fat diet control. HFD-GBH: high-fat diet + glyphosate-based herbicide.

3.2 Morphological and morphometric analysis of muscle fibers

The standard morphological structure was preserved in most muscle fibers of the diaphragm in both groups, i.e., polygonal multinucleate cells with peripheral nuclei that were arranged in fascicles (Figure 2A and 2B). However, some areas in the muscle contained fibers with large nuclei exhibiting a basophilic halo (Figure 2C and 2D) and central nucleoli (Figure 2E and 2F). Many rounded and hypereosinophilic muscle fibers were also observed in the two groups studied (Figure 3A and 3B), as well as fibers with destructured myofibrils in the cytoplasm (vacuolization) (Figure 3C and 3D).

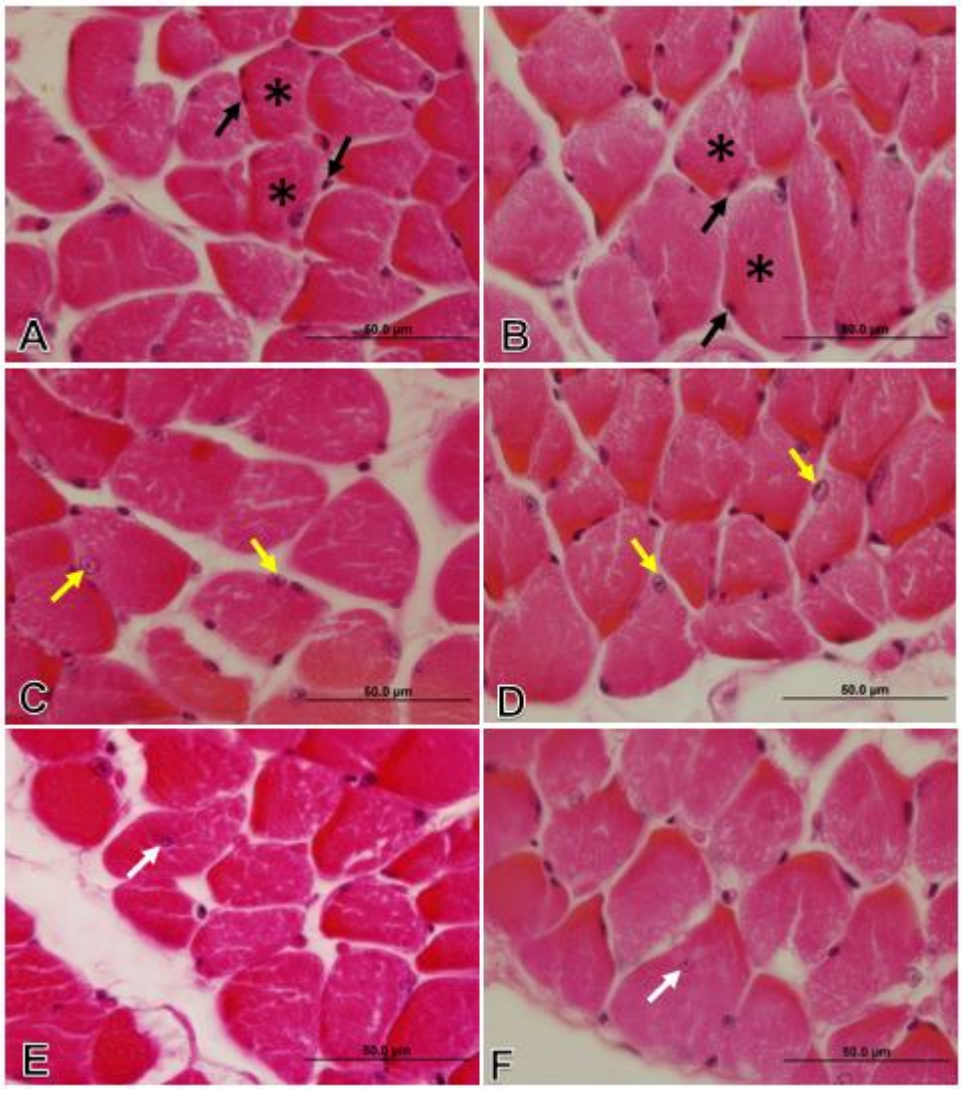

Figure 2. Photomicrographs of the diaphragm muscle of C57BL/6 mice at 150 days of age. Cross-section, hematoxylin-eosin staining. High-fat diet control group (A, C, and E). High-fat diet + glyphosate-based herbicide group (B, D, and F). (A) and (B), polygonal muscle fibers

(*) with peripheral nuclei (black arrow). (C) and (D), large nuclei with a basophilic halo (yellow arrow). (E) and (F), central nuclei (white arrow).

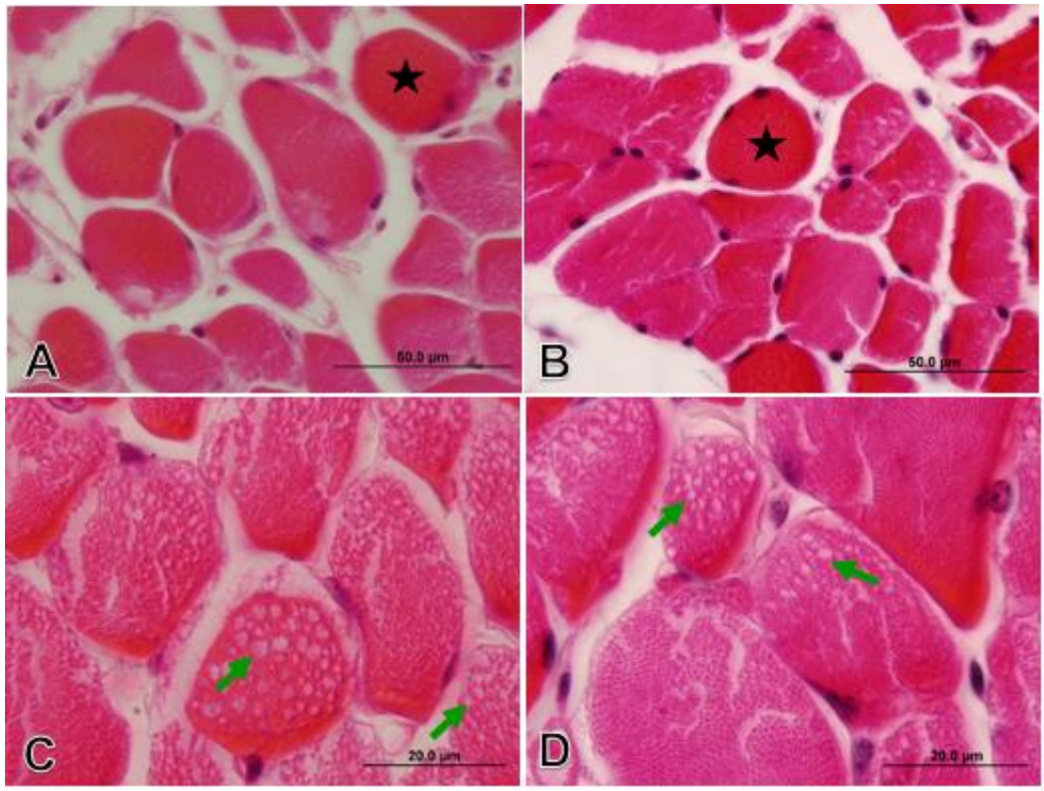

Figure 3. Photomicrographs of the diaphragm muscle of C57BL/6 mice at 150 days of age. Cross-section, hematoxylin-eosin staining. High-fat diet control group (A and C). High-fat diet + glyphosate-based herbicide group (B and D). (A) and (B), rounded and hypereosinophilic muscle fibers (star). (C) and (D), muscle fibers with vacuolized cytoplasm (green arrow).

Muscle fiber splitting (longitudinal division of the muscle fiber) (Figure 4A and 4B), degenerating muscle fibers, some atrophic fibers (Figure 4C), and other hypertrophic and hypereosinophilic fibers with homogenous cytoplasm without striations (hyalinization) (Figure 4D) were observed exclusively in the HFD-GBH group.

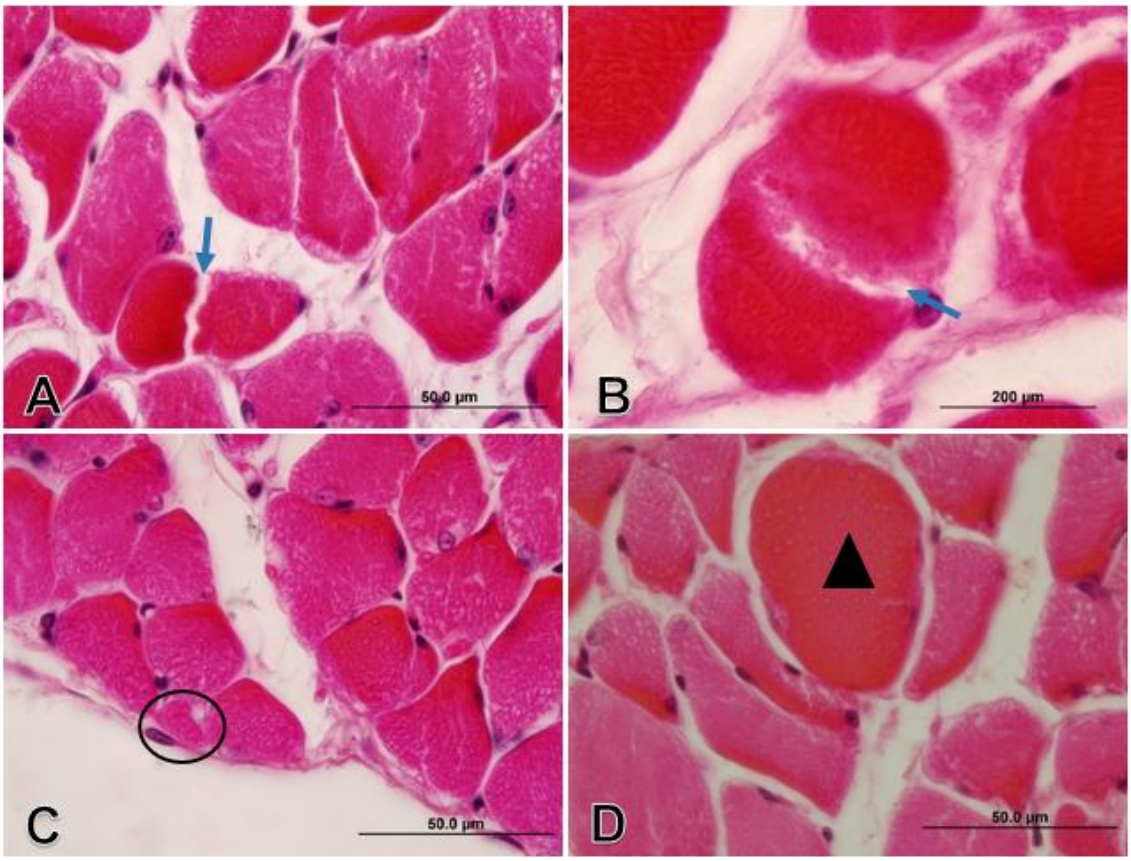

Figure 4. Photomicrographs of the diaphragm muscle of C57BL/6 mice at 150 days of age. Cross-section, hematoxylin-eosin staining. High-fat diet + glyphosate-based herbicide group. (A) and (B), muscle fiber splitting (blue arrow). (C), atrophied muscle fiber (ellipse). (D), hypertrophic and hypereosinophilic fiber (triangle).

Regarding the morphometric characteristics of diaphragm muscle fibers, no significant difference was observed between the two groups in any of the parameters analyzed (Table 2).

Table 2. Morphometric analysis of diaphragm muscle fibers of C57BL/6 mice at 150 days of age in the high-fat diet control group and high-fat diet + glyphosate-based herbicide group.

Morphometric parameters	Groups		P value
	HFD-CTL	**HFD-GBH**	
Fiber área (µm²)	885.15 ± 123.82	770.02 ± 186.06	0.3411
Maximum diameter (µm)	41.99 ± 3.38	38.20 ± 5.17	0.2692
Minimum diameters (µm)	25.04 ± 1.73	23.28 ± 2.58	0.2747
Number of fibers	953.20 ± 153.02	937.60 ± 207.81	0.9106

Peripheral nuclei	1376 ± 188.71	1516.80 ± 202.94	0.2824
Central nuclei	43.20 ± 13.48	40.20 ± 4.71	0.6004
Capillar	541 ± 99.33	591.20 ± 70.74	0.4870
Nuclei/fiber ratio	1.45 ± 0.17	1.64 ± 0.17	0.2148
Capillar/fiber ratio	0.57 ± 0.08	0.64 ± 0.08	0.1494

Values are expressed as the mean ± standard deviation. Kolmogorov-Smirnov test. Student *t*-test ($p < 0.05$). HFD-CTL: high-fat diet control. HFD-GBH: high-fat diet + glyphosate-based herbicide.

3.3 Morphological and morphometric analysis of NMJs

The NMJs found in the diaphragm of the animals studied were polymorphic, exhibiting an oval, round or elliptic shape. No morphological changes were observed (Figure 5A and 5B).

Morphometric analysis showed an increase in the area and maximum diameter of NMJs in the HFD-GBH group compared to the HFD-CTL group (Figure 5C and 5D). There was no significant difference in minimum diameter between groups (Figure 5E).

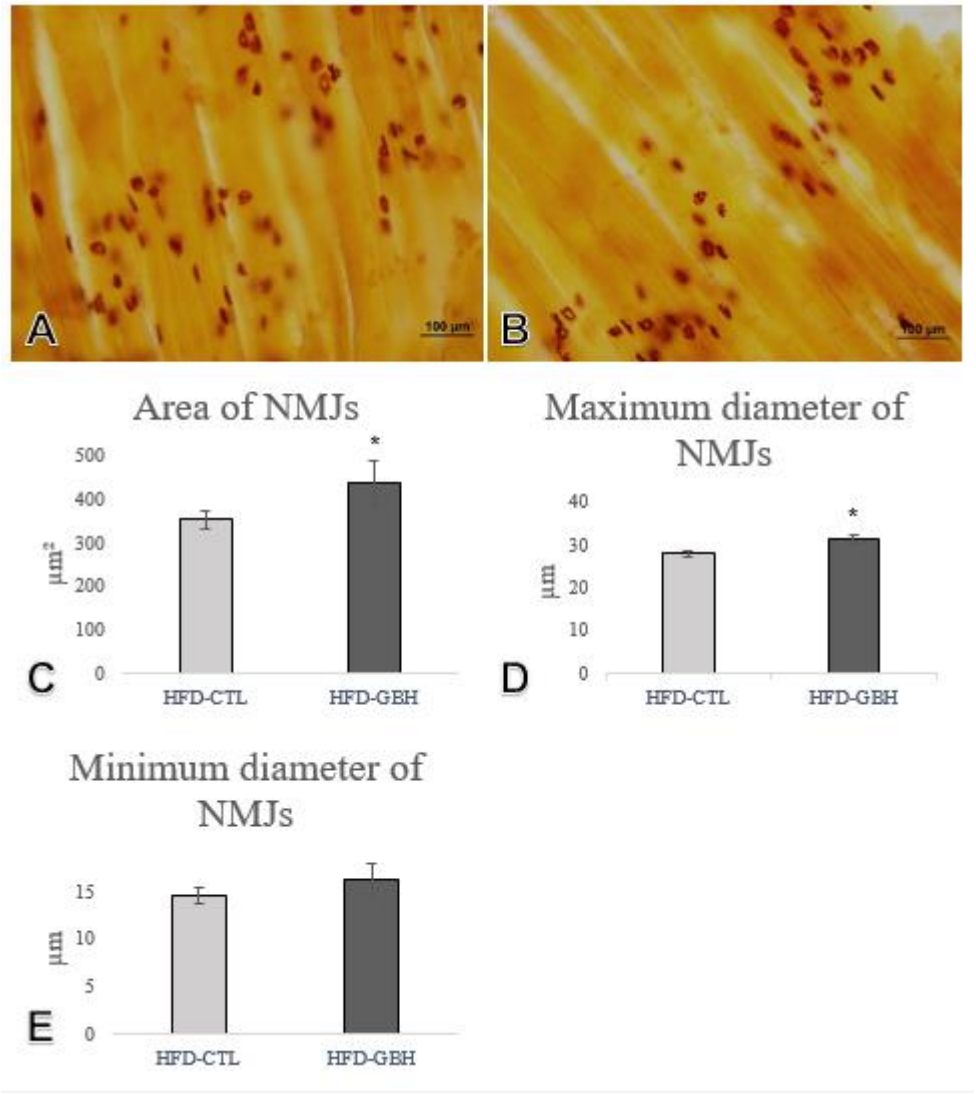

Figure 5. Photomicrographs of neuromuscular junctions (NMJs) in the diaphragm muscle of C57BL/6 mice at 150 days of age. Longitudinal section, nonspecific esterase reaction. High-fat diet control group (HFD-CTL) (A). High-fat diet + glyphosate-based herbicide group (HFD-GBH) (B). (A) and (B), polymorphic NMJs showing no morphological changes. (C), (D) and (E) show the area, maximum diameter and minimum diameter of NMJs, respectively. The asterisk indicates a significant difference ($p < 0.05$). Kolmogorov-Smirnov test. Student *t*-test.

4. Discussion

According to the literature, individual insults do not necessarily promote significant changes in adulthood but the combination of two insults at different stages may explain the emergence of some pathologies in the future. In other words, genetic susceptibility combined

with an insult occurring at some stage of development (first-hit) can lead to the reorganization of different organ systems, which alone may be insufficient to alter the adult phenotype. However, a “second-hit” caused by new insults at a later stage in life can result in endocrine alterations that are sufficient to reveal unseen damage or even to amplify it, resulting in pathologies (Padmanabhan et al., 2016). Hence, in the present study, the exposure to glyphosate during the peripubertal period was considered the first-hit and the induction of obesity by the HFD in adulthood the second-hit.

Peripubertal exposure to GBH from 30 to 60 days of age did not exacerbate the body weight of male adult mice with HFD-induced obesity. Romano et al. (2008) also found no body weight changes in male rats receiving doses of Roundup Transorb of 5, 50 and 250 mg/kg live weight by gavage from days 23 to 53. According to Zhao et al. (2021), oral treatment with glyphosate in water for 4 weeks also did not alter the body weight of male mice. The present results agree with the literature showing that glyphosate was unable to alter the body weight of male mice.

Still regarding body weight, Kubsad et al. (2019) observed important generational toxicology when pregnant rats were exposed to glyphosate (25 mg/kg) for 14 days during gestation. The results showed body weight loss at weaning in the F1 generation in both females and males, while there were no body weight changes in the F2 generation. In the F3 generation, only male rats exposed to glyphosate exhibited body weight gain. In addition, an increase in the frequency of obesity was observed in the F2 and F3 generations. The authors concluded that direct exposure has low toxicity and that glyphosate promotes transgenerational epigenetic inheritance (Kubsad et al., 2019). Within this context, the present results confirm that direct exposure to GBH during the peripubertal period does not cause body weight changes in adulthood. However, the literature shows that the endocrine disruption potential of glyphosate can modify metabolism and thus interfere with body weight and predisposition to obesity in future generations (Kubsad et al., 2019).

Yan et al. (2021) observed gastrocnemius muscle atrophy in obese mice induced by feeding the animals a HFD for 3 months. Another recent study on obese male rats induced by a hypercaloric diet for 16 weeks showed muscle fiber degeneration, increased fat accumulation, and recruitment of macrophages in the biceps femoris muscle. Small and rounded fibers and hypertrophic fibers were also found. According to the authors, these fibers are the result of repeated cycles of muscle degeneration and regeneration (Asghar et al.,

2021). The findings of the present study corroborate literature data showing that obesity induced by a HFD can cause degenerative lesions in diaphragm muscle fibers. Furthermore, the diaphragm is considered to be the main respiratory muscle (Cavaliere et al., 1996) and obesity is known to cause chronic respiratory overload (Espino-Gonzalez et al., 2021). Thus, the choice of the studied muscle (diaphragm) may have also influenced the myopathic changes found in obese mice.

Areas containing muscle fibers with altered morphology were detected in the diaphragms of the HFD-GBH and HFD-CTL groups. The nuclei had a larger size. The concept of nuclear domain refers to the fact that the nucleus controls a finite portion of the sarcoplasm (Hikida et al., 2000). Increased sarcolemma nuclei are indicative of an early regenerative response (Crabbs, 2021), increasing the protein synthesis rate (Santos et al., 2019). Thus, the enlarged volume of myonuclei, together with the other results of this study, demonstrate that the muscle fibers underwent a regeneration process in order to minimize the damage caused by fiber degeneration induced by obesity alone and by obesity associated with GBH.

In addition to enlarged nuclei, mice of the two groups exhibited many rounded and hypereosinophilic fibers as well as vacuolization of the cytoplasm. Rounded muscle fibers suggest myopathy (Camargo Filho et al., 2006), as do vacuolization and fiber division. Such alterations also occurred in skeletal muscle after systemic administration of agents with direct myotoxic effects (Greaves et al., 2013).

Taken together, the results suggest that obesity induced by a HFD alone promoted toxic effects on skeletal muscle. When the HFD was combined with GBH (HFD-GBH group), some myopathic features were only found in this group, which can be explained by the myotoxicity of pesticides, as demonstrated by Cavaliere et al. (1996) who reported muscle fiber necrosis in the diaphragm of rats poisoned with sublethal doses of the organophosphate paraoxon. Bright et al. (1991) administered a sublethal dose of the organophosphate sarin to mice and observed muscle fiber degeneration and mononuclear infiltration, preceded by calcium accumulation.

Obese adult mice exposed to GBH from 30 to 60 days of life exhibited fibers with a longitudinal division of the cytoplasm (splitting). This morphophysiological adaptation of segmentation (internal or lateral) of the fiber is aimed at favoring metabolic exchanges and at preventing injuries by reducing the distance between the capillary network and cytoplasm

(Ciabattari et al., 2005). Studies have shown that these alterations can be triggered by different process such as stress resulting from exercise, metabolic stress, and microcirculatory changes (Camargo Filho et al., 2006). We therefore believe that this division of the muscle fiber is the result of attempted cell recovery after damage caused by the stress arising from the combination with GBH at a critical stage of development and the obesity in adulthood.

Atrophic, hypertrophic and hypereosinophilic fibers were found in the HFD-GBH group. The enlargement of some muscle fibers observed in this study can be explained by compensatory hypertrophy, which is characterized by concomitant atrophic changes in the muscle (Greaves et al., 2013), i.e., in response to the atrophy of some fibers, there was an overload of other fibers, inducing fiber hypertrophy. Atrophic fibers also indicate a degenerative process. Atrophy resulting from non-neurogenic causes, such as the administration of myotoxic chemicals, is generally characterized by the presence of myopathic changes such as rounded fibers, cells with central nuclei, and fibers undergoing splitting (Greaves et al., 2013).

Analysis of NMJs showed an increase in area and maximum diameter in the HFD-GBH group when compared to the HFD-CTL group. It is important to remember that glyphosate is an organophosphate herbicide that acts by inhibiting acetylcholinesterase (Barbosa et al., 2021; Meek et al., 2021). Thus, the degradation of acetylcholine is slow and this compound consequently remains in the synaptic cleft for a longer period of time, enhancing cholinergic transmission (Araújo et al., 2016). Although we did not analyze acetylcholinesterase activity in this study, it is possible that GBH, inhibited acetylcholinesterase in the muscles of mice of the HFD-GBH group, which resulted in the accumulation of acetylcholine and consequent overstimulation of the postsynaptic membrane at the NMJ. This process may have changed the area and the maximum diameter of NMJs.

4. Conclusion

The results of the present study show that HFD-induced obesity promotes degenerative changes in the diaphragm muscle fibers of adult mice. Peripubertal exposure to GBH from 30 to 60 days of age exacerbates the myotoxic effects of obesity.

Conflict of interest declaration

The authors declare that they have no conflict of interest.

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References

Araújo, C.R.M., Santos, V.L.A., Gonsalves, A.A., 2016. Acetilcolinesterase - AChE: Uma Enzima de Interesse Farmacológico. Rev. Virtual Quim. 8, 1818–1834. https://doi.org/10.21577/1984-6835.20160122

Asghar, A., Akhtar, T., Batool, T., Babar, M., Nadeem, S., Mehmood, R., Sheikh, N., 2021. High-fat diet-induced splenic, hepatic, and skeletal muscle architecture damage: cellular and molecular players. Mol. Cell. Biochem. 476, 3671–3679. https://doi.org/10.1007/s11010-021-04190-6

Bai, S.H., Ogbourne, S.M., 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ. Sci. Pollut. Res. 23, 18988–19001. https://doi.org/10.1007/s11356-016-7425-3

Baillie-Hamilton, P.F., B, M., D.Phil, B.S., 2002. Chemical Toxins: A Hypothesis to Explain the Global Obesity Epidemic. J. Altern. Complement. Med. 8, 185–192. https://doi.org/10.1089/107555302317371479.

Barbosa, A., Oliveira, M.C., Kuhn-fraga, C., Balbo, S.L., Torrejais, M.M., 2021. Estudo das fibras musculares dos músculos extensor longo dos dedos e sóleo de fêmeas C57BL / 6 expostas ao glifosato durante a prenhez e a lactação. Einstein (Sao Paulo) 19, 1–7. https://doi.org/10.31744/einstein_journal/2021AO5657

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Séralini, G.E., 2007. Time- and Dose-Dependent Effects of Roundup on Human Embryonic and Placental Cells. Arch Env. Contam Toxicol. 53, 126–133. https://doi.org/10.1007/s00244-006-0154-8

Bernardis, L.L., Patterson, B.D., 1968. Correlation Between “Lee Index” And Carcass Fat Content In Weanling And Adult Female Rats With Hypothalamic Lesions. J. Endrocr. 40, 527–528. https://doi.org/10.1677/joe.0.0400527

Boudalia, S., Bousbia, A., Boumaaza, B., Oudir, M., Chantal, M., Lavier, C., 2021. Relationship between endocrine disruptors and obesity with a focus on bisphenol A: a narrative review. Bioimpacts. 11, 289–300. https://doi.org/10.34172/bi.2021.33

Bright, J.E., Inns, R.H., Tuckwell, N.J., Griffiths, G.D., Marrs, T.C., 1991. A Histochemical

Study of Changes Observed in the Mouse Diaphragm after Organophosphate Poisoning. Hum. Exp. Toxicol. 10, 9–14.

Camargo Filho, J.C.S., Vanderlei, L.C.M., Camargo, R.C.T., Oliveira, D.A.R., Oliveira Júnior, S.A., Dal Pai, V., Belangero, W.D., 2006. Análise histológica, histoquímica e morfométrica do músculo sóleo de ratos submetidos a treinamento físico em esteira rolante. Arq. Ciênc. Saúde 12, 196–199.

Cavaliere, M.J., Calore, E.E., Perez, N.M., Puga, F.R., 1996. Miotoxicidade por organofosforados. Rev. Saúde Pública 30, 267–272. https://doi.org/10.1590/S0034-89101996000300010

Cerf, M.E., Louw, J., Herrera, E., 2015. High Fat Diet Exposure during Fetal Life Enhances Plasma and Hepatic Omega-6 Fatty Acid Profiles in Fetal Wistar Rats. J. Nutr. 7, 7231–7241. https://doi.org/10.3390/nu7095337

Ciabattari, O., Dal Pai, A., Dal Pai, V., 2005. Efeito da natação associado a diferentes dietas sobre o músculo tibial anterior do rato: estudo morfológico e histoquímico. Rev. Bras. Med. Esporte 11. https://doi.org/10.1590/S1517-86922005000200005

Crabbs, T.A., 2021. NTP Nonneoplastic Lesion Atlas: Skeletal Muscle - Degeneration. [internet]. URL https://ntp.niehs.nih.gov/nnl/musculoskeletal/skel_musc/degen/skeletal-muscle-degeneration_508.pdf (accessed 12.4.21).

Espino-gonzalez, E., Tickle, P.G., Benson, A.P., Askew, G.N., Egginton, S., Bowen, T.S., 2021. Abnormal skeletal muscle blood flow, contractile mechanics and fibre morphology in a rat model of obese-HFpEF. J. Physiol. 3, 981–1001. https://doi.org/10.1113/JP280899

Fernandes, G.S.A., Arena, A.C., Campos, K.E., Volpato, G.T., Anselmo-franci, J.A., Damasceno, D.C., Kempinas, W.G., 2012. Glutamate-induced obesity leads to decreased sperm reserves and acceleration of transit time in the epididymis of adult male rats. Reprod. Biol. Endocrinol. 10, 105. https://doi.org/10.1186/1477-7827-10-105

George, J., Prasad, S., Mahmood, Z., Shukla, Y., 2010. Studies on glyphosate-induced carcinogenicity in mouse skin: A proteomic approach. J Proteomics. 73, 951–964. https://doi.org/10.1016/j.jprot.2009.12.008

Gluckman, P.D., Hanson, M.A., Mitchell, M.D., 2010. Developmental origins of health and disease: reducing the burden of chronic disease in the next generation. Genome Med. 2, 4–6. https://doi.org/10.1186/gm135

Greaves, P., Chouinard, L., Ernst, H., Mecklenburg, L., Pruimboom-brees, I.M., Rinke, M., Rittinghausen, S., Thibault, S., Erichsen, J. Von, Yoshida, T., 2013. Proliferative and Non-Proliferative Lesions of the Rat and Mouse. J. Toxicol. Pathol. 26, 1S–26S. https://doi.org/10.1293/tox.26.1S

Hikida, R.S., Staron, R.S., Hagerman, F.C., Walsh, S., Kaiser, E., Shell, S., Hervey, S., 2000. Effects of High-Intensity Resistance Training on Untrained Older Men. II. Muscle Fiber Characteristics and Nucleo-Cytoplasmic Relationships. J. Gerontol. 55A, B347–B354. https://doi.org/10.1093/gerona/55.7.b347.

Ho, S., Cheong, A., Adgent, M.A., Veevers, J., Suen, A.A., Tam, N.N.C., Leung, Y., Jefferson, W.N., Williams, C.J., 2016. Environmental factors, epigenetics, and developmental origin of reproductive disorders. Reprod. Toxicol. 68, 85–104. https://doi.org/10.1016/j.reprotox.2016.07.011

Ingaramo, P., Alarcón, R., Muñoz-de-Toro, M., Luque, E.H., 2020. Are glyphosate and glyphosate-based herbicides endocrine disruptors that alter female fertility? Mol. Cell. Endocrinol. 518, 110934. https://doi.org/10.1016/j.mce.2020.110934

Ivanski, F., Oliveira, V.M., Oliveira, I.M., Ramos, A.T.A., Tonete, S.T.O., Hykavei, G.O., Bargi-Souza, P., Schiessel, D.L., Martino-Andrade, A.J., Romano, M.A., Romano, R.M., 2020. Prepubertal acrylamide exposure causes dose-response decreases in spermatic production and functionality with modulation of genes involved in the spermatogenesis in rats. Toxicology. 436, 152428. https://doi.org/10.1016/j.tox.2020.152428

Junqueira, L.C., Carneiro, J., 2013. Histologia Básica, 12th ed. Guanabara Koogan, Rio de Janeiro.

Kalofiri, P., Balias, G., Tekos, F., 2021. The EU endocrine disruptors' regulation and the glyphosate controversy. Toxicol. Rep. 8, 1193–1199. https://doi.org/10.1016/j.toxrep.2021.05.013

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. 27, 1A-149A.

Kubsad, D., Nilsson, E.E., King, S.E., Sadler-riggleman, I., Beck, D., Skinner, M.K., 2019. Assessment of Glyphosate Induced epigenetic transgenerational Inheritance of pathologies and sperm epimutations: Generational toxicology. Sci. Rep. 9, 6372. https://doi.org/10.1038/s41598-019-42860-0.

Lehrer, G.M., Ornstein, L., 1959. A diazo coupling method for the electron microscopic localization of cholinesterase. J. Biophys. Biochem. Cytol. 6, 399–419. https://doi.org/10.1083/jcb.6.3.399.

Li, G., Liu, J.Y., Zhang, H.X., Li, Q., Zhang, S.W., 2015. Exercise Training Attenuates Sympathetic Activation and Oxidative Stress in Diet-Induced Obesity. Physiol. Res. 64, 355–367. https://doi.org/10.33549/physiolres.932851

Lucaccioni, L., Trevisani, V., Marrozzini, L., Bertoncelli, N., Predieri, B., Lugli, L., Berardi, A., Iughetti, L., 2020. Endocrine-Disrupting Chemicals and Their Effects during Female Puberty: A Review of Current Evidence. Int. J. Mol. Sci. 21, 2078. https://doi.org/10.3390/ijms21062078

Marques, E.B., Souza, K.P., Alvim-Silva, T., Martins, I.L.F., Pedro, S., Scaramello, C.B. V., 2021. Nutrition and Cardiovascular Diseases: Programming and Reprogramming. Int. J. Cardiovasc. Sci. 34, 197–210. https://doi.org/10.36660/ijcs.20200031

Meek, E.C., Carr, R.L., Chambers, J.E., 2021. Toxicology in Vitro In vitro age-related differences in rats to organophosphates. Toxicol. Vitr. 72, 105102. https://doi.org/10.1016/j.tiv.2021.105102

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.E., 2015. Potential toxic

effects of glyphosate and its commercial formulations below regulatory limits. Food Chem. Toxicol. 84, 133–153. https://doi.org/10.1016/j.fct.2015.08.012

Milesi, M.M., Lorenz, V., Durando, M., Rossetti, M.F., Varayoud, J., 2021. Glyphosate Herbicide: Reproductive Outcomes and Multigenerational Effects. Front. Endocrinol. 12, 672532.

Newbold, R.R., Padilla-Banks, E., Jefferson, W.N., Heindel, J.J., 2008. Effects of endocrine disruptors on obesity. Int. J. Androl. 31, 201–208. https://doi.org/10.1111/j.1365-2605.2007.00858.x

Owagboriaye, F., Dedeke, G., Ademolu, K., Olujimi, O., Aladesida, A., Adeleke, M., 2019. Comparative studies on endogenic stress hormones, antioxidant, biochemical and hematological status of metabolic disturbance in albino rat exposed to roundup herbicide and its active ingredient glyphosate. Environ. Sci. Pollut. Res. 26, 14502–14512. https://doi.org/10.1007/s11356-019-04759-1

Padmanabhan, V., Cardoso, R.C., Puttabyatappa, M., 2016. Developmental Programming, a Pathway to Disease. Endocrinology. 157, 1328–1340. https://doi.org/10.1210/en.2016-1003

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere. 61, 1115–1122. https://doi.org/10.1016/j.chemosphere.2005.03.044

Petrovics, P., Sandor, B., Palfi, A., Szekeres, Z., Atlasz, T., Toth, K., Szabados, E., 2021. Association between Obesity and Overweight and Cardiorespiratory and Muscle Performance in Adolescents. Int. J. Environ. Res. Public. Heal. 18, 134. https://doi.org/10.3390/ijerph18010134

Rasslan, Z., Stirbulov, R., Lima, C.A. da C., Júnior, R.S., 2009. Função pulmonar e obesidade*. Rev. Bras. Clin. Med. 7, 36–39.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G., 2005. Differential Effects of Glyphosate and Roundup on Human Placental Cells and Aromatase. Env. Heal. Perspect. 113, 716–720. https://doi.org/10.1289/ehp.7728

Romano, R.M., Romano, M.A., Moura, M.O., Oliveira, C.A., 2008. A exposição ao glifosato-Roundup causa atraso no início da puberdade em ratos machos. Braz. J. vet. Res. anim. Sci. 45, 481–487.

Romano, R.M., Romano, M.A., Oliveira, C.A., 2009. Glifosato como desregulador endócrino químico. Ambiência - Rev. do Set. Ciências Agrárias e Ambient. 5, 359–372.

Santos, V.C., Vechetti Júnior, I.J., Sant'Ana, D.M.G., Almeida, F.L.A., 2019. Vias de sinalizaçãocelular envolvidas no crescimento e manutenção do músculo estriado esquelético. Arq Mudi. 23, 220–248. https://doi.org/10.4025/arqmudi.v23i3.51539

Schimpf, M.G., Milesi, M.M., Ingaramo, P.I., Luque, E.H., Varayoud, J., 2017. Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus. Toxicology. 376, 2–14. https://doi.org/10.1016/j.tox.2016.06.004

Schneider, M., Pons, J., Labesse, G., Bourguet, W., 2019. In Silico Predictions of Endocrine

Disruptors Properties. Endocrinology. 160, 2709–2716. https://doi.org/10.1210/en.2019-00382

Silveira, P.P., Portella, A.K., Goldani, M.Z., Barbieri, M.A., 2007. Origens desenvolvimentistas da saúde e da doença (DOHaD). J. Pediatr. (Rio. J). 83, 494–504. https://doi.org/10.2223/JPED.1728

Sizoo, D., Heide, L.J.M., Emous, M., van Zutphen, T., Navis, G., van Beek, A.P., 2021. Measuring Muscle Mass and Strength in Obesity: a Review of Various Methods. Obes Surg. 31, 384–393. https://doi.org/10.1007/s11695-020-05082-2

Soudani, N., Chaabane, M., Ghorbel, I., Elwej, A., Boudawara, T., Zeghal, N., 2019. Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. Gen. Physiol. Biophys. 38, 123–134.

Suzuki, K., 2017. The developing world of DOHaD. J. Dev. Orig. Health Dis. 1–4. https://doi.org/10.1017/S2040174417000691

Teleken, J.L., Gomes, E.C.Z., Marmentini, C., Moi, M.B., Ribeiro, R.A., Balbo, S.L., Amorim, E.M.P., Bonfleur, M.L., 2020. Glyphosate-based herbicide exposure during pregnancy and lactation malprograms the male reproductive morphofunction in F1 offspring. J. DOHaD. 11, 146–153. https://doi.org/10.1017/S2040174419000382

Thakur, D.S., Khot, R., Joshi, P.P., Pandharipande, M., Nagpure, K., 2014. Glyphosate Poisoning with Acute Pulmonary Edema. Toxicol. Int. 21, 328–331. https://doi.org/10.4103/0971-6580.155389

Walsh, L.P., Mccormick, C., Martin, C., Stocco, D.M., 2000. Roundup Inhibits Steroidogenesis by Disrupting Steroidogenic Acute Regulatory (StAR) Protein Expression. Env. Heal. Perspect 108, 769–776.

WHO/UNEP, 2013. State of the science of endocrine-disrupting chemicals - 2012, in: Bergman, A., Heindel, J.J., Jobling, S., Kidd, A.K., Zoeller, R.T. United Nations Environment Programme and the World Health Organization, Geneva, p. 296.

Yan, X., Niu, Q., Gao, X., Shen, S., He, N., Wang, H., Fang, R., Gao, Y., Chang, H., 2021. Differential Protein Metabolism and Regeneration in Gastrocnemius Muscles in High-fat Diet Fed Mice and Pre-hibernation Daurian Ground Squirrels: A Comparison between Pathological and Healthy Obesity. Zool. Stud. 60, 6. https://doi.org/10.6620/ZS.2021.60-06

Yilmaz, B., Terekeci, H., Sandal, S., Kelestimur, F., 2020. Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. Rev. Endocr. Metab. Disord. 21, 127–147. https://doi.org/10.1007/s11154-019-09521-z.

Zhao, L., Zhang, J., Yang, L., Zhang, H., Zhang, Y., Gao, D., Jiang, H., Li, Y., Dong, H., Ma, T., Wang, X., Wu, M., Wang, A., Jin, Y., Yuan, Y., HuataoChen, 2021. Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells. Sci Total Environ. 785, 147323. https://doi.org/10.1016/j.scitotenv.2021.147323

CONCLUSÕES FINAIS

A exposição peripuberal ao GBH altera a morfometria das fibras musculares do diafragma de camundongos adultos, altera a morfometria das JNMs do músculo diafragma de camundongos adultos obesos e não obesos e exacerba as alterações degenerativas da fibra muscular do diafragma de camundongos adultos obesos. Portanto, os resultados demonstram que o GBH no período peripuberal é capaz de promover alterações nas fibras musculares e JNMs do diafragma de camundongos adultos, obesos ou não obesos.

REFERÊNCIAS GERAIS

ABRASCO. Associação Brasileira de saúde coletiva. Dossiê ABRASCO: um alerta sobre os impactos dos agrotóxicos na saúde. Rio de Janeiro/São Paulo, 2015.

ABESO. Associação Brasileira para o Estudo da Obesidade e da Síndrome Metabólica. Diretrizes brasileiras de obesidade. 2016, 4.ed. Brasília: ABESO, 2016.

AMARANTE JUNIOR, O. P. et al. Glifosato: propriedades, toxicidade, usos e legislação. Química Nova, v. 25, n. 4, p. 589–593, 2002.

ANDRADE, A., PINTO, S. C., OLIVEIRA, R. S. Animais de Laboratório: criação e experimentação [online]. Rio de Janeiro: Editora FIOCRUZ, 2002. p. 388. Disponível em: <https://books.scielo.org/id/sfwtj/pdf/andrade-9788575413869-16.pdf>.

ASGHAR, A. et al. High-fat diet-induced splenic, hepatic, and skeletal muscle architecture damage: cellular and molecular players. Molecular and Cellular Biochemistry, v. 476, n. 10, p. 3671–3679, 2021.

BAI, S. H.; OGBOURNE, S. M. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environmental Science and Pollution Research, v. 23, n. 19, p. 18988–19001, 2016.

BAILLIE-HAMILTON, P. F. Chemical Toxins: A Hypothesis to Explain the Global Obesity Epidemic. Journal of Alternative and Complementary Medicine, v. 8, n. 2, p. 185–192, 2002.

BARBOSA, E. R. et al. Parkinsonism after glycine-derivate exposure. Movement Disorders, v. 16, n. 3, p. 565–568, maio 2001.

BARBOSA, J. E. S. et al. Influência do músculo diafragma no controle postural, na propriocepção e na dor lombar. Revista Brasileira de Fisiologia do Exercício, v. 18, n. 4, p. 236–246, 2019.

BARBOZA, H. T. G. et al. Compostos Organofosforados e seu Papel na Agricultura. Revista Virtual de Química, v. 10, n. 1, p. 172–193, 2018.

BAROUKI, R. et al. Developmental origins of non-communicable disease: Implications for research and public health. Environmental Health, v. 11, p. 42, 2012.

BIOLO, G.; CEDERHOLM, T.; MUSCARITOLI, M. Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: From sarcopenic obesity to cachexia. Clinical Nutrition, v. 33, n. 5, p. 737–748, 2014.

BLAKEMORE, S.; BURNETT, S.; DAHL, R. E. The Role of Puberty in the Developing Adolescent Brain. Human Brain Mapping, v. 31, n. 6, p. 926–933, 2010.

BOUDALIA, S. et al. Relationship between endocrine disruptors and obesity with a focus on bisphenol A: a narrative review. Bioimpacts, v. 11, n. 4, p. 289–300, 2021.

BRIGHT, J. E. et al. A Histochemical Study of Changes Observed in the Mouse Diaphragm after Organophosphate Poisoning. Human & Experimental Toxicology, v. 10, p. 9–14, 1991.

CALORE, E. E. et al. Sarcoplasmic lipase and non-specific esterase inhibition in myofibers of rats intoxicated with the organophosphate isofenphos. Experimental and Toxicologic Pathology, v. 51, n. 1, p. 27–33, 1999.

CAMPOS, Y. et al. Exposure to pesticides and mental disorders in a rural population of Southern Brazil. NeuroToxicology, v. 56, p. 7–16, 2016.

CARGNELUTTI, F. et al. Effects of endocrine disruptors on fetal testis development, male puberty, and transition age. Endocrine, v. 72, n. 2, p. 358–374, 2021.

CAVALCANTI, L. P. A. N. et al. Intoxicação por organofosforados: tratamento e metodologias analíticas empregadas na avaliação da reativação e inibição da acetilcolinesterase. Revista Virtual de Química, v. 8, n. 3, p. 739–766, 2016.

CAVALIERE, M. J. et al. Miotoxicidade por organofosforados. Revista de Saúde Pública, v. 30, n. 3, p. 267–272, 1996.

CERF, M. E; LOUW, J.; HERRERA, E. High Fat Diet Exposure during Fetal Life Enhances Plasma and Hepatic Omega-6 Fatty Acid Profiles in Fetal Wistar Rats. Journal Nutrients, v. 7, p. 7231–7241, 2015.

CVITIC, S. et al. Human fetoplacental arterial and venous endothelial cells are differentially programmed by gestational diabetes mellitus, resulting in cell-specific barrier function changes. Diabetologia, v. 61, n. 11, p. 2398–2411, 2018.

DAL-PAI-SILVA, M.; CARVALHO, R. F. Mecanismos celulares e moleculares que controlam o desenvolvimento e o crescimento muscular. Revista Brasileira de Zootecnia, v. 36, p. 21–31, 2007.

DALLEGRAVE, E. et al. The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats. Toxicology Letters, v. 142, p. 45–52, 2003.

DUTTA, S.; SENGUPTA, P. Men and mice: Relating their ages Sulagna. Life Sciences, v. 152, p. 244–248, 2016.

ERIGUCHI, M. et al. Parkinsonism relating to intoxication with glyphosate. Internal Medicine, v. 58, p. 1935-1938, 2019.

ESPINOZA-NAVARRO, O.; PONCE-LAROSA, C.; BUSTOS-OBREGÓN, E. Organophosphorous Pesticides: Their Effects on Biosenti- nel Species and Humans. Control and Application in Chile. International Journal of Morphology, v. 35, n. 3, p. 1069–1074, 2017.

FAGERLUND, M. J.; ERIKSSON, L. I. Current concepts in neuromuscular transmission. British Journal of Anaesthesia, v. 103, n. 1, p. 108–114, 2009.

FERLONI, A. et al. Exposición fetal a bisfenol a: presencia de bisfenol a en orina de mujeres gestantes asistidas en un hospital de la ciudad de buenos aires. Año 2013. Revista de la Facultad de Ciencias Médicas de Córdoba, v. 76, n. 2, p. 86–91, 2019.

FERNANDES, L. S. Neuropatia retardada induzida por organofosforados: estudo in vitro dos mecanismos de neurotoxicidade do organofosforado triclorfom e estratégias de neuroproteção. 2017. 148 f. Tese (Doutorado em Ciências) - Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2017.

FUDVOYE, J.; LOPEZ-RODRIGUEZ, D.; PARENT, A. S. Endocrine disrupters and possible contribution to pubertal changes. Best Practice & Research Clinical Endocrinology & Metabolism, v. 33, n. 3, p. 101300, 2019.

GANU, R. S. et al. Maternal diet: a modulator for epigenomic regulation during development in nonhuman primates and humans. International Journal of Obesity Supplements, v. 2, p. 14–18, 2012.

GARTNER, L. P.; HIATT, J. L. Tratado de Histologia em cores. 3. ed. Rio de Janeiro: Elsevier, 2007.

GIAQUINTO, P. C. et al. Effects of Glyphosate-Based Herbicide Sub-Lethal Concentrations on Fish Feeding Behavior. Bulletin of Environmental Contamination and Toxicology, v. 98, n. 4, p. 460–464, 2017.

GILL, J. P. K. et al. Glyphosate toxicity for animals. Environmental Chemistry Letters, v. 16, n. 2, p. 401–426, 2018.

GLUCKMAN, P. D. et al. Effect of In Utero and Early-Life Conditions on Adult Health and Disease. The New England journal of medicine, v. 359, n. 14, p. 61–73, 2008.

GLUCKMAN, P. D; HANSON, M. A; MITCHELL, M. D. Developmental origins of health and disease: reducing the burden of chronic disease in the next generation. Genome Medicine, v. 2, n. 14, p. 4–6, 2010.

GREENSPAN, L. C.; LEE, M. M. Endocrine Disrupters and Pubertal Timing Louise. Journal of Endocrinology, Diabetes & Obesity, v. 25, n. 1, p. 49–54, 2019.

GUYTON, K. Z. et al. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. The Lancet Oncology, v. 16, n. 5, p. 490–491, 2015.

HO, S. et al. Environmental factors, epigenetics, and developmental origin of reproductive disorders. Reproductive Toxicology, v. 68, p. 85–104, 2016.

HOLOVSKÁ, K. et al. Effect of the bendiocarb on the ultrastructure of rabbit skeletal muscle. Acta Veterinaria Brno, v. 86, p. 219–222, 2017.

HSU, S. S.; JAN, C. R.; LIANG, W. Z. Uncovering malathion (an organophosphate insecticide) action on Ca2+ signal transduction and investigating the effects of BAPTA-AM (a cell- permeant Ca2+ chelator) on protective responses in glial cells. Pesticide Biochemistry and Physiology, v. 157, p. 152–160, 2019.

INGARAMO, P. et al. Are glyphosate and glyphosate-based herbicides endocrine disruptors that alter female fertility?. Molecular and Cellular Endocrinology, v. 518, n. 3, p. 110934, 2020.

JUNQUEIRA, L. C.; CARNEIRO, J. Histologia Básica. 12. ed. Rio de Janeiro: Guanabara Koogan, 2013.

KUNZ, R. I. et al. Efeitos da imobilização e remobilização pela combinação natação e salto em meio aquático, sobre a morfologia do músculo tibial anterior de ratos. Publicatio UEPG: Ciências Biológicas e da Saúde, v. 19, n. 2, p. 123–129, 2013.

LAKER, R. C. et al. Epigenetic origins of metabolic disease: The impact of the maternal condition to the offspring epigenome and later health consequences. Food Science and Human Wellness, v. 2, n. 1, p. 1–11, 2013.

LANE, R. H. Fetal programming, epigenetics, and adult onset disease. Clinics in Perinatology, v. 41, n. 4, p. 815–831, 2014.

LUCACCIONI, L. et al. Endocrine-Disrupting Chemicals and Their Effects during Female Puberty: A Review of Current Evidence. International Journal of Molecular Sciences, v. 21, n. 6, p. 2078, 2020.

MAGALHÃES, A. F. A. Avaliação das intoxicações exógenas no Distrito Federal no período de 2009 a 2013. 2017. 151 f. Tese (Doutorado em Ciências da Saúde) - Universidade de Brasília, Brasília, 2017.

MAO, Q. et al. The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome. Environmental Health, v. 17, n. 50, p. 1–12, 2018.

MARQUES, E. B. et al. Nutrition and Cardiovascular Diseases: Programming and Reprogramming. International Journal of Cardiovascular Sciences, v. 34, n. 2, p. 197–210, 2021.

MARQUES JUNIOR, N. K. Mecanismos Fisiológicos da Fadiga. Revista Brasileira de Prescrição e Fisiologia do Exercício, v. 9, n. 56, p. 671–720, 2015.

MESNAGE, R. et al. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food and Chemical Toxicology, v. 84, p. 133–153, 2015.

MONNERET, C. What is an endocrine disruptor?. Comptes Rendus Biologies, v. 340, p. 403–405, 2017.

MOON, J.; CHUN, B.; LEE, S. Variable response of cholinesterase activities following human exposure to different types of organophosphates. Human and Experimental Toxicology, v. 34, n. 7, p. 698–706, 2015.

MYERS, J. P. et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environmental Health, v. 15, p. 19, 2016.

NEVES, P. D. M.; BELLINI, M. Intoxicações por agrotóxicos na mesorregião norte central paranaense, Brasil – 2002 a 2011. Revista Ciência & Saúde Coletiva, v. 18, n. 11, p. 3147–3156, 2013.

NEWBOLD, R. R. et al. Effects of endocrine disruptors on obesity. International Journal of Andrology, v. 31, p. 201–208, 2008.

OJEDA, S. R.; SKINNER, M. K. Puberty in the Rat. Knobil and Neill's Physiology of Reproduction, p. 2061–2126, 2006.

OLARTE, L. C. et al. Revista Brasileira de Prescrição e Fisiologia do Exercício. Revista Brasileira de Prescrição e Fisiologia do Exercício, v. 11, n. 64, p. 110–121, 2017.

OLIVEIRA, M. C. et al. Whole-Body Vibration , Morphological and Antioxidant Effects on the Diaphragm Muscle of Obese Rats. Muscles, Ligaments and Tendons Journal, v. 11, n. 4, p. 648–656, 2021.

OSUNA-FLORES, I. et al. Effect of organophosphorus pesticides in juveniles of Litopenaeus vannamei: alteration of glycogen, triglycerides, and proteins. Ecotoxicology, 2019.

OWAGBORIAYE, F. et al. Comparative studies on endogenic stress hormones, antioxidant, biochemical and hematological status of metabolic disturbance in albino rat exposed to roundup herbicide and its active ingredient glyphosate. Environmental Science and Pollution Research, v. 26, n. 14, p. 14502–14512, 2019.

PADMANABHAN, V.; CARDOSO, R. C.; PUTTABYATAPPA, M. Developmental Programming, a Pathway to Disease. Endocrinology, v. 157, n. 4, p. 1328–1340, 2016.

PANDEY, A.; DHABADE, P.; KUMARASAMY, A. Inflammatory Effects of Subacute Exposure of Roundup in Rat Liver and Adipose Tissue. Dose-Response, v. 17, n. 2, p. 1–11, 2019.

PARKER, K. L.; SCHIMMER, B. P. Embryology and genetics of the mammalian gonads and ducts. In: Knobil Neill's Physiol. Reprod. 3. ed. Londres: Elsevier, 2006. p. 313–336.

PERVANIDOU, P.; CHROUSOS, G. P. Metabolic consequences of stress during childhood and adolescence. Metabolism, v. 61, n. 5, p. 611–619, 2012.

PETROVICS, P. et al. Association between Obesity and Overweight and Cardiorespiratory and Muscle Performance in Adolescents. International Journal of Environmental Research and Public Health, v. 18, p. 134, 2021.

PUNGA, A. R.; RUEGG, M. A. Signaling and aging at the neuromuscular synapse: Lessons learnt from neuromuscular diseases. Current Opinion in Pharmacology, v. 12, n. 3, p. 340–346, 2012.

RASSLAN, Z. et al. Função pulmonar e obesidade*. Revista da Sociedade Brasileira de Clínica Médica, v. 7, n. 1, p. 36–39, 2009.

REYNOSO, E. C. et al. Trends and Perspectives in Immunosensors for Determination of Currently-Used Pesticides: The Case of Glyphosate, Organophosphates, and Neonicotinoids. Biosensors, v. 9, p. 20, 2019.

RIBEIRO, C. T. et al. Evaluation of the effects of chronic stress applied from the prepubertal to the adult stages or only during adulthood on penile morphology in rats. The International Journal on the Biology of Stress, v. 22, n. 2, p. 248–255, 2019.

ROMANO, R. M. et al. A exposição ao glifosato-Roundup causa atraso no início da puberdade em ratos machos. Brazilian Journal of Veterinary Research and Animal Science, v. 45, n. 6, p. 481–487, 2008.

ROMANO, R. M.; ROMANO, M. A.; OLIVEIRA, C. A. Glifosato como desregulador endócrino químico. Revista Ambiência, v. 5, n. 2, p. 359–372, 2009.

SAMANTA, P. et al. Histopathological and ultrastructural alterations in some organs of Oreochromis niloticus exposed to glyphosate-based herbicide, Excel Mera 71. Journal of Microscopy and Ultrastructure, v. 6, n. 1, p. 35–43, 2018.

SAMSEL, A.; SENEFF, S. Glyphosate, pathways to modern diseases III: Manganese, neurological diseases, and associated pathologies. Surgical Neurology International, v. 6, p. 45, 2015.

SANTAMARTA, J. A ameaça dos disruptores endócrinos*. Revista Agroecologia e Desenvolvimento Rural Sustentável, v. 2, n. 3, p. 18–29, 2001.

SANTOS-JÚNIOR, F. F. U. et al. Alterações Morfométricas em Músculo Respiratório de Ratos Submetidos à Imobilização de Pata. Revista Brasileira Medicina do Esporte, v. 16, n. 3, p. 215–218, 2010.

SCHIMPF, M. G. et al. Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus. Toxicology, v. 376, p. 2–14, 2017.

SERRA, L. S. et al. Revolução Verde: reflexões acerca da questão dos agrotóxicos. Revista Cientifica do Centro de Estudos em Desenvolvimento Sustentável da UNDB, v. 1, n. 4, p. 2–25, 2016.

SIDDEEK, B. et al. Sperm epigenome as a marker of environmental exposure and lifestyle, at the origin of diseases inheritance. Mutation Research - Reviews in Mutation Research, v. 778, p. 38–44, 2018.

SILVA, J. P. et al. Interferências da programação metabólica no desenvolvimento da obesidade e suas comorbidades. Salus Journal of Health Sciences, v. 1, n. 1, p. 91–99, 2015.

SILVA, K. N. et al. Músculos respiratórios: fisiologia, avaliação e protocolos de treinamento. Revista Cereus, v. 6, p. 1–13, 2012.

SILVA, M. R.; CAMPOS, A. C. E.; BOHM, F. Z. Agrotóxicos e seus impactos sobre ecossistemas aquáticos continentais. Revista de Saúde e Biologia, v. 8, n. 2, p. 46–58, 2013.

SILVEIRA, P. P. et al. Origens desenvolvimentistas da saúde e da doença (DOHaD). Jornal de Pediatria, v. 83, n. 6, p. 494–504, 2007.

SILVÉRIO, A. C. P. et al. Assessment of exposure to pesticides in rural workers in southern of Minas Gerais, Brazil. Environmental Toxicology and Pharmacology, v. 55, p. 99–106, 2017.

SIQUEIRA, S. L.; KRUSE, M. H. L. Agrotóxicos e saúde humana: contribuição dos profissionais do campo da saúde. Revista da Escola de Enfermagem da USP, v. 42, n. 3, p. 584–590, 2008.

SOARES, W.; ALMEIDA, R. M. V. R.; MORO, S. Trabalho rural e fatores de risco associados ao regime de uso de agrotóxicos em Minas Gerais, Brasil. Caderno de saúde pública, v. 19, n. 4, p. 1117–1127, 2003.

SOUCHARD, P. O diafragma. 4. ed. São Paulo: Summus Editorial, 1989.

SOUDANI, N. et al. Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. General Physiology and Biophysics, v. 38, p. 123–134, 2019.

SOUZA, I. C.; GONÇALVES, A. S. Computational Studies of Fullerene Derivatives as Pesticide Captors. Orbital: The Electronic Journal of Chemistry, v. 11, n. 1, p. 10–17, 2019.

SPYKER, J. M.; AVERY, D. L. Neurobehavioral effects of prenatal exposure to the organophosphate Diazinon in mice. Journal of Toxicology and Environmental Health, v. 3, p. 989–1002, 1977.

SUZUKI, K. The developing world of DOHaD. Journal of Developmental Origins of Health and Disease, p. 1–4, 2017.

TALBOT, J.; MAVES, L. Skeletal muscle fiber type: using insights from muscle developmental biology to dissect targets for susceptibility and resistance to muscle disease. Wiley Interdisciplinary Reviews: Developmental Biology, v. 5, n. 4, p. 518–534, 2016.

TAPIA-MARTÍNEZ, J. et al. Maternal Thyroid Hormone Deficiency During Gestation and Lactation Alters Metabolic and Thyroid Programming of the Offspring in the Adult Stage. Hormone and Metabolic Research, v. 51, n. 6, p. 381–388, 2019.

TEJERINA, G. R. L. Intoxicações e óbitos por agrotóxicos no Estado de Goiás, Brasil e inovações legislativas. Cadernos Ibero-Americanos De Direito Sanitário, v. 7, n. 1, p. 229–249, 2018.

TIZHE, E. V. et al. Influence of zinc supplementation on histopathological changes in the stomach, liver, kidney, brain, pancreas and spleen during subchronic exposure of Wistar rats to glyphosate. Comparative Clinical Pathology, v. 23, p. 1535–1543, 2014.

TORREJAIS, M. M. et al. Morphometric and Morphological Analysis of Neuromuscular Junction Alterations in the Denervated Rat Diaphragm. International Journal of Morphology, v. 27, n. 4, p. 1235–1242, 2009.

TSOORY, M. M.; GUTERMAN, A.; RICHTER-LEVIN, G. '"Juvenile Stress"' Alters Maturation-Related Changes in Expression of the Neural Cell Adhesion Molecule L1 in the Limbic System: Relevance for Stress-Related Psychopathologies. Journal of Neuroscience Research, v. 88, p. 369–380, 2010.

VALE, D. B. J. et al. A utilização de organofosforados na agricultura brasileira: riscos para a saúde humana e danos ambientais - uma revisão bibliográfica. Convibra, 2017.

WANDERLEY, E. N.; FERREIRA, V. A. Obesidade: uma perspectiva plural. Ciência & Saúde Coletiva, v. 15, n. 1, p. 185–194, 2010.

WHO/UNEP. State of the science of endocrine-disrupting chemicals - 2012. 2013, Geneva: United Nations Environment Programme and the World Health Organization, 2013. p. 296.

WILKENS, A. L. L.; VALGAS, A. A. N.; OLIVEIRA, G. T. Effects of ecologically relevant concentrations of Boral® 500 SC, Glifosato® Biocarb, and a blend of both herbicides on markers of metabolism, stress, and nutritional condition factors in bullfrog tadpoles. Environmental Science and Pollution Research, v. 26, n. 23, p. 23242–23256, 2019.

YAN, X. et al. Differential Protein Metabolism and Regeneration in Gastrocnemius Muscles in High-fat Diet Fed Mice and Pre-hibernation Daurian Ground Squirrels: A Comparison between Pathological and Healthy Obesity. Zoological Studies, v. 60, p. 6, 2021.

ZHU, R. et al. Oxidative stress and liver disease. Hepatology Research, v. 42, n. 8, p. 741–749, 2012.

ANEXO 01 - Parecer do Cômite de Ética

unioeste
Universidade Estadual do Oeste do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação
Comitê de Ética no Uso de Animais - CEUA

Autorização

O protocolo intitulado **"Efeitos do herbicida glifosato sobre a homeostase glicêmica e lipídica em camundongos obesos pela dieta hiperlipídica"**, sob a responsabilidade de **Maria Lucia Bonfleur** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata*, para fins de pesquisa científica encontra-se **Aprovado** para execução, está de acordo com as Normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pelo Comitê de Ética no Uso de Animais (CEUA) do UNIOESTE em reunião de 11/05/2018.

FINALIDADE	Pesquisa Científica
Vigência da autorização	01/06/2018 - 30/04/2020
Espécie/linhagem/raça	Camundongos: *Mus musculus*, camundongo isogênico C57BL/6.
N. de animais	144
Peso/idade	45-20g/30 dias
Sexo	Masculino
Origem	Biotério Central da Universidade Estadual do Oeste do Paraná - UNIOESTE - Campus Cascavel - PR.

Cascavel, 15/05/2018

Profa. Dra. Luciana Oliveira de Fariña
Coordenadora do CEUA
Portaria nº 3730/2016 - GRE

ANEXO 02 - Normas para submissão Revista Tissue and Cell

GUIDE FOR AUTHORS

INTRODUCTION

Types of paper

Tissue and Cell is devoted to original research on the organization of **cells**, **subcellular** and **extracellular components** at all levels, including the grouping and interrelations of cells in **tissues** and **organs**. The journal encourages submission of ultrastructural studies that provide novel insights into structure, function and physiology of cells and tissues, in health and disease. Bioengineering and stem cells studies focused on the description of morphological and/or histological data are also welcomed.

Studies investigating the effect of compounds and/or substances on structure of cells and tissues are generally outside the scope of this journal. For consideration, studies should contain a clear rationale on the use of (a) given substance(s), have a compelling morphological and structural focus and present novel incremental findings from previous literature.

Four types of papers are welcome: full length article, method articles, short communications and review articles.

Full Length Articles are devolved to description of original research studies. There are no limits in length and display items (figures and tables).

Method Articles should describe new technological advancements or provide comparative evidence between new methods and pre-existing protocols. The focus of the paper should be in describing the technical steps in sufficient detail to be replicated. Where appropriate, authors should guide the reader through the procedure and provide expected observations/results alongside the protocol.

Short Communications are short papers intended to provide quick publication of timely and impactful results. Short Communications are expected to report a single scientific conclusion that represent a novel discovery in the field. Short communications should be no longer than 2000 words and contain max 2 figures.

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Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
- E-mail address
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All necessary files have been uploaded:
Manuscript:
- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
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Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

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• Manuscript has been 'spell checked' and 'grammar checked'
• All references mentioned in the Reference List are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
• A competing interests statement is provided, even if the authors have no competing interests to declare
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Ethics in publishing

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If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

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All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and **the authors should clearly indicate in the manuscript that such guidelines have been followed pending manuscript editorial rejection**. During the upload process, an ARRIVE checklist is required for animal studies and animal experiments. Manuscripts lacking checklists will be returned to authors for completion.

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