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Aléxia dos Reis

Ototoxicidade induzida pela exposição a agrotóxicos: estudo em modelo animal.

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Ototoxicidade induzida pela exposição a agrotóxicos: estudo em modelo animal.

Tese submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutora.

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Resumo da Tese

Introdução: O uso de agrotóxicos isolados e em combinação é muito comum na agricultura, bem como na saúde pública a fim de combater vetores de doenças e na medicina veterinária para eliminar ectoparasitas. Embora diversas pesquisas já tenham apontado os impactos negativos na saúde de indivíduos expostos a tais substâncias, a ototoxicidade secundária a organofosforados e piretróides, classes de agrotóxicos muito utilizados, ainda é pouco explorada, sendo necessários novos estudos para estabelecer se há nexo-causal e possíveis mecanismos de toxicidade. **Objetivos:** Avaliar os efeitos do organofosforado (OF) diclorvós, do piretróide (PIR) cipermetrina e da associação de ambos na função auditiva de ratos por meio de emissões otoacústicas produto de distorção (EOAPDs) e potenciais evocados auditivos de tronco encefálico (PEATE). **Material e Métodos:** dois projetos foram realizados. No primeiro estudo, 36 ratos Wistar foram divididos entre 3 grupos (12 ratos/grupo): controle negativo (água), controle positivo para lesão auditiva (8 mg/kg de cisplatina) e grupo experimental (associação de 1,48 mg/L de diclorvós e 0,25 mg/L de cipermetrina; OF + PIR). A exposição ocorreu de forma inalatória por 4 h, 5 vezes por semana, durante 6 semanas (grupos controle negativo e exposição). O grupo controle positivo recebeu cisplatina via intraperitonial durante 3 dias consecutivos, 24 h antes da eutanásia. Foram avaliadas emissões otoacústicas evocadas produto de distorção e variáveis de toxicidade sistêmica (massa corporal, sinais clínicos, níveis de colinesterase plasmática e massa relativa dos órgãos). No segundo estudo, 25 ratos foram divididos em cinco grupos (5 ratos/grupo): controle negativo (água), controle positivo para lesão auditiva (8 mg/kg de cisplatina), OF (1,48 mg/L), PIR (0,25

mg/L) e grupo OF + PIR (associação de 1,48 mg/L de diclorvós e 0,25 mg/L de cipermetrina). A exposição ocorreu de forma inalatória por 4 h, 5 vezes por semana, durante 6 semanas (grupos controle negativo, OF, PIR e OF + PIR). O grupo controle positivo recebeu cisplatina via intraperitonial durante 3 dias consecutivos, 24 h antes da eutanásia. Foram avaliadas emissões otoacústicas evocadas produto de distorção, PEATE e variáveis de toxicidade sistêmica (massa corporal, sinais clínicos, níveis de colinesterase plasmática e massa relativa dos órgãos). **Resultados:** No primeiro estudo, não houve diferença estatisticamente significativa entre os grupos nos parâmetros de massa corporal, sinais clínicos ou nos níveis de colinesterase plasmática. Houve redução de amplitudes das EOAPDs no grupo experimental. No segundo projeto, não houve diferença estatisticamente significativa entre os grupos nos parâmetros de massa corporal, sinais clínicos ou nos níveis de colinesterase plasmática. O grupo cisplatina apresentou redução na mediana das amplitudes das EOAPDs pós exposição em 4 kHz, 10 kHz e 12 kHz. O grupo PIR apresentou redução na mediana das amplitudes das EOAPDs pós exposição em 6 kHz e 8 kHz e no grupo OF + PIR houve redução em 12 kHz. No teste PEATE, o grupo cisplatina apresentou uma mediana maior bilateralmente quando comparado ao grupo controle negativo. À esquerda, o grupo OF + PIR apresentou mediana menor quando comparado ao grupo controle positivo para lesão auditiva. O grupo OF+ PIR apresentou bilateralmente resultados similares ao grupo controle negativo (água). **Conclusão:** Houve ototoxicidade secundária à exposição aos agrotóxicos avaliados por EOAPD e PEATE, sem toxicidade sistêmica relevante.

Palavras-chave: ratos; agrotóxicos; audição; toxicidade.

Abstract

Introduction: The use of pesticides alone and in combination is very common in agriculture, as well as in public health in order to combat disease vectors and in veterinary medicine to eliminate ectoparasites. Although several studies have already pointed out the negative impacts on the health of individuals exposed to such substances, ototoxicity secondary to organophosphates and pyrethroids, classes of widely used pesticides, is still little explored. Further studies are needed to establish whether there is a causal link and possible mechanisms of toxicity. **Aim of study:** To evaluate the effects of organophosphate (OF) dichlorvos, pyrethroid (PIR) cypermethrin and their association on the auditory function of rats through distortion product otoacoustic emissions (DPOAEs) and brainstem electric response audiometry (BERA). **Materials and methods:** Two projects were carried out. In the first study, 36 Wistar rats were divided into 3 groups (12 rats/group): negative control (water), positive control for hearing damage (8 mg/kg of cisplatin) and experimental group (association of 1.48 mg/L of dichlorvos and 0.25 mg/L of cypermethrin; OP + PYR). The animals were exposed subchronically by inhalation for 4 hours, 5 times a week, during 6 weeks (negative control and exposure groups). The positive control group received intraperitoneal cisplatin for 3 consecutive days, 24 hours before euthanasia. Distortion product otoacoustic emissions and systemic toxicity variables (body mass, clinical signs, plasma cholinesterase levels and relative organ mass) were evaluated. In the second study, 25 rats were divided into five groups (5 rats/group): negative control (water), positive control for hearing damage (8 mg/kg of cisplatin), OP (1.48 mg/L), PYR (0.25 mg/L) and OP + PYR group (association of 1.48 mg/L of dichlorvos and 0.25 mg/L of cypermethrin).

The animals were exposed subchronically by inhalation for 4 hours, 5 times a week, during 6 weeks (negative control, OP, PYR and OP + PYR groups). The positive control group received intraperitoneal cisplatin for 3 consecutive days, 24 hours before euthanasia. Distortion product otoacoustic emissions, BERA and systemic toxicity variables (body mass, clinical signs, plasma cholinesterase levels and relative organ mass) were evaluated. **Results:** In the first study, there was no statistically significant difference in body mass parameters, clinical signs, or plasma cholinesterase levels in the experimental group. There was a reduction in DPOAEs amplitudes in the experimental group. In the second project, there was no statistically significant difference in body mass parameters, clinical signs, or plasma cholinesterase levels in the experimental groups. The cisplatin group presented lower DPOAEs medians post exposure in 4 kHz, 10 kHz and 12 kHz. The PYR group had lower amplitudes in 6 kHz and 8 kHz and the association group showed lower amplitudes in 12 kHz. At BERA test on both ears, the cisplatin group presented a higher median when compared to the water group. On the left, the association group had lower median in relation to the cisplatin group. Bilaterally the association group presented similar means to the water group. **Conclusion:** There was secondary ototoxicity to the exposure of the evaluated pesticides assessed by DPOAEs and BERA, without relevant systemic intoxication.

Keywords: rats; pesticides; hearing; toxicity.

Lista de abreviaturas

ABR: *auditory brainstem response.*

ACh: acetilcolina.

AChE: acetilcolinesterase.

BAEPS: *brainstem auditory evoked potentials.*

BAER: *brainstem auditory evoked response.*

BERA: *brainstem electric response audiometry.*

CCE: células ciliadas externas.

EOAPD: emissões otoacústicas produto de distorção.

EOAs: emissões otoacústicas.

EPIs: equipamentos de proteção individual.

GABA: ácido gama-aminobutírico.

ms: milissegundos.

NR7: norma reguladora 7.

OF: organofosforado.

PEATE: potenciais evocados auditivos de tronco encefálico.

PIR: piretroide.

SNC: sistema nervoso central.

SUMÁRIO

1. INTRODUÇÃO

Pesticidas são um grupo de substâncias que têm um importante papel na sociedade, uma vez que aumentam a produção de alimentos e diminuem os índices de patologias causadas por vetores[1]. No entanto, pesquisas têm discutido amplamente seus efeitos, pois tais substâncias representam um risco significativo ao meio ambiente e à saúde de seres humanos[2] .

De acordo com o Relatório Anual de Atendimento do Centro de Informação Toxicológica do Rio Grande do Sul[3], ocorreram 577 atendimentos em humanos no ano de 2019 devidos à exposição a agrotóxicos em geral e 329 por exposição a inseticidas domésticos. Dentre os agrotóxicos de forma geral, a maior parte dos atendimentos adveio de acidentes individuais (337 casos), seguidos de tentativa de suicídio (157 casos) e em terceiro lugar acidentes ocupacionais (76 casos). Nos casos de inseticidas domésticos, 287 ocorrências foram por acidentes individuais e 32 tentativas de suicídio. Especificamente em relação às classes de inseticidas mais envolvidas nas exposições, pode-se citar os organofosforados (50 e 32) e piretróides (123 e 305) de uso agrícola e doméstico, respectivamente[3].

Embora alguns dos efeitos na saúde dos organofosforados (OF) e piretróides (PIR) já tenham sido bem explorados por pesquisas prévias[4-6], outros parâmetros - tais como alterações na audição - ainda necessitam de maior atenção, considerando a escassez de estudos acerca do dano auditivo secundário à exposição a esses agentes[7], bem como seus mecanismos associados.

2. REFERENCIAL TEÓRICO

2.1 Agrotóxicos

De acordo com a Lei[8] Nº 7.802, de 11 de Julho de 1989, regulamentada pelo Decreto[9] nº 4.074, entende-se por agrotóxicos e afins os produtos e agentes de processos físicos, químicos ou biológicos, com destinação ao uso em setores de produção, armazenamento e beneficiamento de produtos agrícolas, pastagens, proteção de florestas (nativas ou plantadas), bem como de outros ecossistemas e ambientes urbanos, hídricos e industriais cuja finalidade seja alterar a composição da flora ou da fauna. Essa alteração tem como objetivo a preservação da flora e fauna da ação danosa de seres vivos considerados nocivos. Também são agrotóxicos as substâncias e produtos empregados como desfolhantes, dessecantes, estimuladores e inibidores de crescimento.

O uso de agrotóxicos no Brasil e em países tropicais é amplo, uma vez que pragas que reduzem a produtividade e prejudicam a aparência de frutas e hortaliças se adaptam melhor a esse clima, causando um dano ainda maior na produção de alimentos nesses países do que em locais que possuem o clima temperado. Nesse sentido, os agrotóxicos – especialmente quando utilizados em ambientes com condição climática favorável ao desenvolvimento de tais pragas - diminuem perdas qualitativas e quantitativas na agricultura, o que mantém a sustentabilidade econômica do produtor[10], aumenta a eficiência da produção e reduz o custo dos alimentos[11]. Também é importante destacar o papel de inseticidas domésticos, frequentemente compostos por piretróides, no combate a vetores[1] como o *Aedes aegypti*, que propaga doenças como a dengue, febre amarela, febre zica e *chikungunya*.

Dentre as principais classes de inseticidas estão os organofosforados e piretróides. Os pesticidas organofosforados são ésteres fosfóricos inicialmente desenvolvidos como armas químicas na Alemanha Nazista, tais como o Sarin, Soman e Tabun[12,13], enquanto os piretroides são substâncias sinteticamente derivadas da piretrinas, que por sua vez são inseticidas naturais que derivam do crisântemo[14].

2.1.1 *Organofosforados*

O mecanismo de ação dos organofosforados envolve a inibição da enzima acetilcolinesterase (AChE) - responsável pela hidrolisação do neurotransmissor acetilcolina (ACh) – através da fosforilação do sítio esterásico[12]. Além disso, a exposição aos OFs pode levar ao estresse oxidativo, déficits de transporte axonal, neuroinflamação e autoimunidade[15]. Essa classe de pesticidas é facilmente absorvida por inalação, via dérmica e por ingestão[16].

Independentemente da inibição da acetilcolinesterase, organofosforados podem fosforilar uma enzima presente no tecido neural denominada esterase-alvo das neuropatias, causando uma progressiva desmielinização de nervos. A polineuropatia retardada induzida por organofosforados ocorre quando a lesão causada pela desmielinização dos nervos é associada à paralisia e degeneração axonal. A polineuropatia pode se apresentar inicialmente por sensação de queimação e formigamento principalmente na área dos pés, com perda de força motora após alguns dias, que também pode afetar pernas e mãos. Ainda pode ocorrer dificuldade em marcha e ataxia, com alteração em sistema nervoso central e autônomo[12].

Ademais, em intoxicações graves por organofosforados pode ocorrer a síndrome intermediária, em que há disfunção da transmissão neuromuscular e insuficiência cardíaca devido à diminuição progressiva da transmissão na junção neuromuscular. Progressivamente pode ocorrer perda da força de músculos respiratórios. Sendo assim, tais pacientes apresentam risco de insuficiência cardiorrespiratória, que pode eventualmente levar a óbito[12].

A intoxicação por agentes organofosforados pode gerar sinais e sintomas da síndrome colinérgica, como miose, lacrimejamento, salivação, sudorese, vômitos, diarreia, broncorreia, broncoconstrição, bradicardia, hipotensão, dor abdominal e incontinência urinária (sistema nervoso parassimpático – receptor muscarínico); taquicardia e hipertensão (sistema nervoso simpático – receptor nicotínico); fasciculações, fraqueza muscular, hiporreflexia (placa neuromuscular – receptor nicotínico); e, no sistema nervoso central, pode causar convulsões, depressão respiratória, rebaixamento do nível de consciência, agitação, confusão mental, ataxia, coma[17].

Além de tais sinais e sintomas de toxicidade secundária à exposição a organofosforados, um estudo pregresso demonstrou ototoxicidade avaliada por emissões otoacústicas por produto de distorção (EOAPD) em ratos Wistar expostos a essa substância, o que indica dano de células ciliadas e na função coclear[7].

O manejo do paciente intoxicado por organofosforados busca a estabilização do paciente. Deve envolver intubação orotraqueal precoce em caso de rebaixamento de nível de consciência ou insuficiência respiratória, uso de cristalóides ou drogas vasoativas - se necessário - na hipotensão e benzodiazepínicos na ocorrência de convulsões. O paciente deve ser

descontaminado, retirando-se a roupa e lavando a pele em água corrente em caso de exposição tópica. O carvão ativado pode ser considerado na dose máxima de 100 mg em até uma hora após a exposição por via oral, contudo o benefício é incerto. A atropina deve ser administrada de forma endovenosa de forma precoce, uma vez que é uma droga que compete pelos receptores muscarínicos e reverte os seus efeitos. A dose e forma de administração da atropina é *in bolus* 1-4 mg (0,05-0,1 mg/kg em crianças) a cada 2-15 minutos até o momento em que os efeitos respiratórios como broncorreia e depressão respiratória cessem. Após a administração *in bolus,* deve-se realizar a bomba de infusão de atropina contínua até a reversão dos efeitos tóxicos[17].

Para tratar as manifestações nicotínicas da toxicidade por OF, deve-se utilizar oximas como a pralidoxima (em geral em intoxicações moderadas ou graves). Administra-se pralidoxima endovenosa *in bolus* 30 mg/kg em 30 minutos e após realiza-se infusão contínua de 8 mg/kg/h até 12 horas após o cessamento dos sintomas colinérgicos[17].

Após a exposição a organofosforados, também pode ocorrer uma síndrome denominada polineuropatia retardada, neuropatia tardia ou neurotóxica tardia, de difícil resolução. Trata-se de uma neuropatia sensitivo-motora que ocorre de forma ascendente nas extremidades dos membros inferiores e superiores em até 4 semanas após a exposição ao agrotóxico. Os sintomas iniciam com formigamento e queimação em dedos que se prolonga aos membros superiores, enquanto há fraqueza e ataxia em membros inferiores. A exposição crônica também pode causar sintomas como cefaléia, alteração na memória e sono, fadiga, inapetência, desorientação e alterações psíquicas[18].

Embora muito frequentes, as intoxicações crônicas são de difícil diagnóstico, tornando-se subnotificadas. De acordo Taveira e Albuquerque[19], outros possíveis motivos para a subnotificação de intoxicações podem incluir a baixa procura pela população exposta e a não notificação de casos diagnosticados. A baixa procura aos serviços de saúde pode ser explicada pela recorrência de sintomas que faz com que o indivíduo se habitue às manifestações clínicas após o uso de agrotóxicos. Além disso, ao receio de indicar falhas no uso de Equipamentos de Proteção Individual (EPIs) e de represálias advindas do agronegócio local, uma vez que considerada a relação nexo causal, irregularidades poderiam ser encontradas após acionamento e vistoria da vigilância sanitária[19].

2.1.2 *Piretroides*

O mecanismo de ação dos piretróides (PIR) em insetos resulta na sua morte pelo rompimento do sistema de transporte de íons da membrana dos axônios, paralisando o sistema nervoso, ocorrendo também o prolongamento do influxo de sódio e bloqueio de vias inibidoras[14] (Olson, 2014). Os PIR são moduladores dos canais de sódio, sendo divididos em tipo I (cujo mecanismo envolve principalmente os canais de sódio voltagem-dependentes) e tipo II (onde também atuam nos canais de cloreto voltagem-dependentes). O PIR tipo II liga-se aos receptores inibitórios do ácido gama-aminobutírico (GABA) no Sistema Nervoso Central (SNC), bloqueando os canais de cloro e sua ativação. Dessa forma, ocorre hiperexcitabilidade devido à ausência de inibição sináptica causada pelo GABA. Assim, ambos os PIR tipos I e II prolongam o tempo de despolarização, gerando hiperexcitabilidade[20,21] em espécies alvo e não alvo[22].

Embora a descrição de seus efeitos tóxicos em humanos envolva frequentemente irritação de pele, via aérea e hipersensibilidade (reações anafiláticas como broncoespasmo, edema orofaríngeo e choque), uma vez que os mamíferos metabolizam rapidamente esses compostos[14], o efeito ototóxico já foi evidenciado em ratos Wistar expostos ao PIR cipermetrina[23].

Em casos de ingestão maciça (200 a 500 mL de solução concentrada), pode ocorrer convulsão, coma ou parada respiratória secundária a dano no sistema nervoso central. Nesses quadros, o paciente deverá ser observado de 6 a 8 horas após a ingestão (em busca de sinais de depressão do SNC ou convulsões), descontaminado e tratado de acordo com os sintomas apresentados, uma vez que não existe antídoto específico para a intoxicação por piretróides[14].

2.1.3 Associação de Agrotóxicos

Frequentemente agrotóxicos são combinados ou misturados para a aplicação, especialmente em países desenvolvidos ou em desenvolvimento. Nos países em desenvolvimento, há provável associação entre a exposição a agrotóxicos em mistura com taxas maiores de fatalidade e morbidade[16]. De forma geral, muitos estudos avaliam a toxicidade de apenas uma substância isolada e com uma via de exposição somente, mas na atividade ocupacional ocorrem exposições de substâncias combinadas ou mesmo multiexposição aos agrotóxicos[24].

Quando em associação, agrotóxicos podem gerar efeitos imprevisíveis, uma vez que existe complexidade nas suas interações toxicológicas[25]. Ainda, embora tais substâncias individualmente sejam correlacionadas com desfechos

desfavoráveis no neurodesenvolvimento, seus efeitos sobre o funcionamento sensorial do organismo ainda não estão completamente elucidados[26]. Os inseticidas organofosforados e piretróides costumam estar presentes combinados no meio ambiente, podendo gerar neurotoxicidade antagônica, aditiva ou sinérgica[27]. Arora (2017) analisou a interação entre compostos organofosforados e piretróides, concluindo que a interação entre ambos é altamente sinérgica[27].

Um estudo avaliou os efeitos neurológicos da exposição a longo prazo da mistura de cinco classes de agrotóxicos, incluindo organofosforado e piretróide, em ratos, por via oral. Foram avaliados parâmetros bioquímicos, histológicos e neurocomportamentais, sendo que todos foram afetados pela associação de agrotóxicos de forma dose-dependente. A administração da mistura de agrotóxicos em uma baixa dose promoveu uma resposta adaptativa devido à estimulação do sistema redox. Nos animais expostos a doses altas da mistura, no entanto, houve um efeito negativo nos parâmetros avaliados, provavelmente devido ao Fenômeno de Hormesis[28]. O Fenômeno de Hormesis ocorre quando há dois tipos de respostas biológicas, uma estimulatória e outra inibitória, após a exposição de um organismo a doses diferentes de um agente que causa estresse[29]. Esse estudo indica, portanto, que mesmo quando em doses previstas por limites legais, pode ocorrer dano ao sistema nervoso, uma vez que tais substâncias se comportam de maneira distinta quando associadas[28].

2.2 Norma Reguladora da Saúde Ocupacional no Brasil

Considerando a legislação vigente[30], a Norma Reguladora 7 ou NR7 (1998) estabelece como obrigatória a elaboração e implementação do Programa de Controle Médico e Saúde Ocupacional (PCMSO) por empregadores e instituições que admitam trabalhadores como empregados. O PCMSO busca prevenir, rastrear e diagnosticar precocemente agravos relacionados à atividade ocupacional[30].

De acordo com a NR7 (1998), trabalhadores expostos a ruído devem realizar controle audiométrico com exames de referência e sequenciais[30]. No entanto, a norma não contempla os trabalhadores expostos a agentes químicos. Ainda, os indivíduos expostos no seu ambiente laboral a organofosforados e carbamatos devem realizar exame de sangue para determinar a atividade da colinesterase. Todavia, um estudo prévio[7] realizado em ratos Wistar expostos subcronicamente de forma inalatória a organofosforado demonstrou que, embora tenha havido ototoxicidade, não houve variação entre a pré-exposição e a pós-exposição na variável de colinesterase plasmática, indicando que pode ocorrer dano auditivo mesmo sem alteração nesse biomarcador.

2.3 Dano Auditivo Induzido por Substâncias

A ototoxicidade é secundária à exposição a agentes químicos que causam lesão na cóclea, sendo o dano – na maioria das vezes – gerado por lesão direta das células ciliadas ou por rompimento de mecanismos de homeostase na cóclea[31].

Existe similaridade entre a perda auditiva secundária à exposição a agentes químicos industriais, drogas ototóxicas (como aminoglicosídeos e cisplatina) e a causada por ruído, uma vez que tendem a ser bilaterais simétricas, irreversíveis, em frequências altas e causando dano predominantemente nas células ciliadas cocleares[32].

O dano auditivo pode ser ainda mais significativo quando há associação entre a exposição ao ruído e a substâncias ototóxicas. Um estudo conduzido em uma indústria calçadista no Vale dos Sinos (RS) evidenciou perdas auditivas em 25% (avaliada por audiometria, nas frequências de 2 a 8 kHz no grupo exposto ao ruído e de 3 a 8 kHz no grupo exposto ao ruído e solvente) em relação a indivíduos não expostos a ruído e solvente (tolueno), apesar da indústria apresentar níveis de ruído e tolueno dentro das normas vigentes[33].

2.4 Avaliação Auditiva

2.4.1 Emissões Otoacústicas

Emissões otoacústicas (EOAs) são respostas geradas pela cóclea na forma de energia acústica[34]. Sua presença indica normalidade em relação à resposta ao som do mecanismo receptor pré neural coclear, bem como do mecanismo de orelha média[35]. Se trata de um exame não invasivo que não interfere no modo normal de operação da cóclea, podendo ser realizado repetidas vezes a fim de mensurar respostas[34].

O uso das EOAs também permite uma avaliação do funcionamento mecânico coclear, obtendo informações objetivas acerca das atividades micromecânicas específicas para elementos pré-neurais ou sensoriais do órgão

de Corti, tais como componentes cocleares que estão distais às terminações das fibras nervosas. Nesse sentido, clinicamente e na pesquisa o uso das EOAs se mostra de suma importância, uma vez que muitas disfunções periféricas auditivas se originam em componentes sensoriais da cóclea. Entre as disfunções periféricas auditivas podemos citar a perda auditiva induzida por ruído, ototoxicidade, perdas auditivas hereditárias, entre outras[34].

É importante frisar que as células ciliadas externas (CCE) dispostas no órgão de Corti, se movimentam junto com a membrana basilar, o que amplifica a intensidade de estímulos recebidos, havendo um escape de energia que é captado como EOAs. Portanto, a avaliação por meio de EOAs permite a avaliação das CCE de forma simples e não invasiva, orientando e facilitando a conduta médica[36].

As EOAs podem ser espontâneas ou evocadas. As primeiras são sinais de banda presentes e medidos na ausência de estimulação acústica externa[34,37], enquanto as segundas são evocadas por diferentes tipos de estímulo acústico. As EOAs evocadas se subdividem de acordo com o tipo de estímulo em: transientes, estímulo-frequência e produto de distorção[34,38].

Nas EOAs evocadas transientes, as respostas ocorrem após estimulação acústica de curta duração, sendo clinicamente úteis na detecção de desordens cocleares[34,38]. As EOAs estímulo-frequência são respostas a tons puros, no entanto, não são amplamente utilizadas clinicamente, uma vez que seu tempo de obtenção é maior e o registro oferece dificuldades técnicas[34,38].

As EOAs produto de distorção são respostas evocadas por 2 tons puros primários (f1 e f2), sendo a relação f1/f2 igual a 1,22. Ambos os tons puros possuem frequências sonoras próximas e são apresentados simultaneamente

no teste. Contudo, a cóclea não é capaz de amplificar de forma linear dois estímulos diferentes. Sendo assim, devido a propriedade não linear da cóclea, surge um produto de distorção, que são novas frequências que não estavam presentes no estímulo provocado[34,39].

2.4.2 Potenciais Evocados Auditivos de Tronco Encefálico

Os potenciais evocados auditivos de tronco encefálico (PEATE), também denominados BERA (*brainstem eletric response audiometry*), ABR (auditory *brainstem response)*, BAEPS (*brainstem auditory evoked potentials*) ou ainda BAER (*brainstem* auditory *evoked response*) são originados na cóclea, no nervo auditivo e vias auditivas do tronco encefálico.

Para que tais potenciais sejam gerados, é necessária a apresentação de estímulos sonoros de breve duração e intensidade suficiente[40]. Esses estímulos acústicos gerarão como respostas um potencial polifásico formado por 7 ondas, formadas em até 12 milissegundos (ms), sendo, portanto, um potencial de curta latência ou um potencial auditivo rápido. A origem mais aceita dos potenciais indica: P_I no nervo coclear distal, P_{II} no nervo coclear proximal, P_{III} no núcleo coclear, P_{IV} no núcleo do complexo olivar superior, P_V no núcleo do lemnisco lateral e do colículo inferior e P_{VI} e P_{VII} nos núcleos da radiação talâmica[36].

Os parâmetros avaliados, de forma geral, em humanos no PEATE são: limiar eletrofisiológico, latências absolutas de P_I e P_V e latências interpicos $P_{I\text{-}V}$, $P_{I\text{-}III}$ e $P_{III\text{-}V}$, sendo as respostas consideradas normais: limiar eletrofisiológico baixo, latência absoluta de P_V menor que 5,5 ms e período interpico $P_{I\text{-}V}$ menor que 4 ms[36].

Em ratos, no entanto, acredita-se que o PEATE apresente 4 componentes (I, II, III e IV) que ocorrem em até 6 ms após e estímulo e refletem a atividade normal das seguintes estruturas: na onda I o nervo auditivo, na onda II o núcleo coclear, na onda III o complexo olivar superior e na onda IV lemnisco lateral e/ou colículo inferior[41-43].

2.4.3 Modelos Animais na Avaliação Auditiva

O uso de modelos animais em pesquisas na área da saúde é vasto, uma vez que muitos temas – em especial envolvendo toxicologia – são de difícil avaliação em seres humanos. Além das questões éticas que impossibilitam a avaliação de determinadas variáveis em seres humanos[44], para avaliar uma variável é necessário que se descartem fatores de confusão que podem contribuir para um mesmo desfecho. No caso de pesquisas que investiguem se agrotóxicos favorecem o surgimento de dano auditivo, o ideal é que outros fatores que também contribuam para tal desfecho (como genética, presbiacusia, uso de drogas ototóxicas, doenças prévias e circunstâncias ambientais como ruído e vibração) não estejam presentes concomitantemente à exposição aos pesticidas. No entanto, torna-se difícil a obtenção de uma amostra significativa de indivíduos que não estejam expostos a mais de um fator de risco para o dano auditivo[45].

Nesse sentido, pesquisas em modelos animais permitem avaliações em ambientes controlados, com linhagens padrão de animais, facilitando a investigação do nexo-causal. Mamíferos em específico possuem diversas vantagens em estudos para avaliação da audição, uma vez que possuem muitas características anatômicas semelhantes às de humanos. Os roedores

são os modelos mais utilizados[45] e o rato, além de apresentar similaridade anatômica e fisiológica com os humanos, é um ótimo modelo para estudos em saúde, uma vez que possui boa adaptabilidade, robustez, boa capacidade de sobrevivência e inteligência[46].

É importante destacar que as pesquisas em modelo animal devem seguir a aplicação do princípio dos 3R's proposto pela Rede de Métodos Alternativos ao uso de animais[47] (RENAMA), que estabelece: Redução (*Reduction*): a amostra deve ser formada pelo menor número possível de animais que proporcione a obtenção das informações necessárias no experimento; Refinamento (*Refinement*): a dor, sofrimento ou estresse do animal experimental deve ser minimizada; e Substituição (*Replacement*): ocorre sem o uso de animais vertebrados em situações em que o nível de informação necessária pode ser adquirido dessa forma.

Em relação aos métodos de avaliação da audição em animais, os exames mais utilizados de acordo com uma revisão de literatura foram as EOAPD e o PEATE. Ambos permitem a caracterização do dano auditivo de forma fidedigna, tratando-se de métodos objetivos e não invasivos[45].

3. REFERÊNCIAS BIBLIOGRÁFICAS

1. Weiss B, Amler S, Amler RW. Pesticides. Pediatrics. 2004;113(4 Suppl):1030-6.
2. Kosamu I, Kaonga C, Utembe W. A Critical Review of the Status of Pesticide Exposure Management in Malawi. Int J Environ Res Public Health. 2020;15;17(18):6727.
3. Rio Grande do Sul. Secretaria Estadual de Saúde. Centro Estadual de Vigilância em Saúde. Centro de Informação Toxicológica do Rio Grande do Sul. Relatório Anual de Atendimento – 2019. Acesso em 08 de março de 2021. Disponível em: https://drive.google.com/file/d/1bA49pURLLZeGRxk4vkCQbXjZ_OttTRIH/view.
4. Chrustek A, Hołyńska-Iwan I, Dziembowska I, Bogusiewicz J, Wróblewski M, Cwynar A, Olszewska-Słonina D. Current Research on the Safety of Pyrethroids Used as Insecticides. Medicina (Kaunas). 2018;28;54(4):61.
5. Hołyńska-Iwan I, Szewczyk-Golec K. Pyrethroids: How They Affect Human and Animal Health? Medicina (Kaunas). 2020;30;56(11):582.
6. Richardson JR, Fitsanakis V, Westerink RHS, Kanthasamy AG. Neurotoxicity of pesticides. Acta Neuropathol. 2019;138(3):343-362.
7. Reis AD, Cunha EO, CostaValle MT, Machado MS, Dallegrave E. Effects of subchronic inhalation exposure to an organophosphorus insecticide compound containing dichlorvos on wistar rats' otoacoustic emissions. Braz J Otorhinolaryngol 2020.
8. Brasil. Presidência da República. Casa Civil. Subchefia para Assuntos Jurídicos. Lei Nº 7.802, de 11 de Julho de 1989.

9. Brasil. Presidência da República. Casa Civil. Subchefia de Assuntos Jurídicos. Decreto Nº 4.074, de 4 De Janeiro De 2002.
10. Lopes CA. É possível produzir alimentos para o Brasil sem agrotóxicos?. Cienc. Cult. 2017;69(4): 52-55.
11. Suratman S, Edwards JW, Babina K. Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. Rev Environ Health. 2015;30(1):65-79.
12. Katzung BG. Farmacologia básica e clínica. 13ª ed. – Porto Alegre: AMGH, 2017.
13. Madsen J. Manual MSD – Versão para profissionais de Saúde. Agentes neurotóxicos de guerra química. 2019. Acesso em 21 de Janeiro de 2021. Disponível em: https://www.msdmanuals.com/pt/profissional/les%C3%B5es-intoxica%C3%A7%C3%A3o/armas-de-destrui%C3%A7%C3%A3o-em-massa/agentes-neurot%C3%B3xicos-de-guerra-qu%C3%ADmica.
14. Olson KR. Manual de toxicologia clínica. 6ª ed. Porto Alegre: AMGH, 2014.
15. Naughton SX, Terry AV Jr. Neurotoxicity in acute and repeated organophosphate exposure. Toxicology. 2018;1;408:101-112.
16. LaDou J. Saúde ocupacional e ambiental internacional. 2016. In: LaDou J and Harrison R. CURRENT / Medicina Ocupacional e Ambiental. 5ª ed. Grupo A.
17. Velasco IT. Medicina de emergência: abordagem prática. 13ª ed. Barueri: Manole, 2019.

18. Caldas LQA. Intoxicações Exógenas Agudas por Carbamatos, Organofosforados, Compostos Bipiridílicos E Piretróides. Centro de Controle de Intoxicações. 2000.
19. Taveira BLS e Albuquerque GSC. Análise das notificações de intoxicações agudas, por agrotóxicos, em 38 municípios do estado do Paraná. Saúde Debate. 2018;42(4):211-222.
20. Dias MB, Prata PHL, Sato AS. 2013. Organoclorados, Piretrinas e Piretroides. In: Filho ADA, Campolina D, Dias MB. Toxicologia na Prática Clínica. 2nd edition. Belo Horizonte: Folium. 519-26.
21. Santos MAT, Areas MA, Reyes FGR. 2007. Piretroides - Uma Visão Geral. Alim. Nutr., Araraquarav.18(3): 339-349.
22. Wolansky MJ, Tornero-Velez R. 2013. Critical Consideration of the Multiplicity of Experimental and Organismic Determinants of Pyrethroid Neurotoxicity: A Proof of Concept, Journal of Toxicology and Environmental Health, Part B. 16(8): 453-490.
23. Cunha EO, Reis AD, Macedo MB, Machado MS, Dallegrave E. Ototoxicity of cypermethrin in Wistar rats. Braz J Otorhinolaryngol 2019;86(5):587-92.
24. Murakami Y, Pinto NF, Albuquerque GSC, Perna PO, Lacerda A. Intoxicação crônica por agrotóxicos em fumicultores. Saúde debate. 2017;41(113):563-576.
25. Hernández AF, Gil F, Lacasaña M. Toxicological interactions of pesticide mixtures: an update. Arch Toxicol 2017;91(10):3211-23.
26. Sturza J, Silver MK, Xu L et al. Prenatal exposure to multiple pesticides is associated with auditory brainstem response at 9months in a cohort study of Chinese infants. Environ Int 2016;92-93:478–85.

27. Arora S, Balotra S, Pandey G, Kumar A. Binary combinations of organophosphorus and synthetic pyrethroids are more potent acetylcholinesterase inhibitors than organophosphorus and carbamate mixtures: An in vitro assessment. Toxicol Lett. 2017;15;268:8-16.
28. Ghasemnejad-Berenji M, Nemati M, Pourheydar B, Gholizadeh S, Karimipour M, Mohebbi I, Jafari A. Neurological effects of long-term exposure to low doses of pesticides mixtures in male rats: Biochemical, histological, and neurobehavioral evaluations. Chemosphere. 2021;264(Pt 2):128464.
29. Veline ED. Hormesis: efeito de subdoses nas aplicações fitossanitárias. Comissão Científica e Temática. Anais do Congresso Brasileiro de Fitossanidade. Universidade Estadual Paulista, Câmpus de Jaboticabal. 2019.
30. Brasil. Portaria nº 19. Secretaria Segurança e Saúde no Trabalho 09/04/1998/ NR7. Programa de Controle médico de Saúde ocupacional. Diretrizes e parâmetros mínimos para avaliação e acompanhamento da audição em trabalhadores expostos a níveis de pressão sonora elevado. (D.O.U. 22/04/1998)
31. Dobie R. 2016. Perda Auditiva. In: LaDou J and Harrison R. CURRENT / Medicina Ocupacional e Ambiental. 5ª ed. Grupo A.
32. Morata TC and Little MB. Suggested guidelines for studying the combined effects of occupational exposure to noise and chemicals on hearing. Noise Health. 2002;4;73-87
33. Sebben et al. Efeitos na audição de trabalhadores expostos ao Tolueno e ruído em indústria calçadista da região do Vale dos Sinos (RS).

Toxicovigilância – Toxicologia Clínica: dados e indicadores selecionados, Rio Grande do Sul – 2008-2009, Porto Alegre. 2009;11-18.

34. Probst R, Lonsbury-Martin BL, and Martin GK. A review of otoacoustic emissions. The Journal of the Acoustical Society of America. 1991;89(5), 2027–2067.
35. Kemp DT, Ryan S, Bray P. A Guide to the effective use of Otoacoustic Emissions. Ear and Hearing. 1990;11(2): 93-105.
36. Filho OL et al. Novo Tratado De Fonoaudiologia. 3ª ed. Barueri: Manole, 2013.
37. Filho OL et al. Transient Evoked and Acoustic Distortion Products Otoacoustic Emissions in Hearing Evaluation of Newborns with Few Horas of Life. Braz J Otorhinolaryngol. 1996;62:220-8.
38. Pialarissi PR e Gattaz G. Emissões Otoacústicas: Conceitos Básicos e Aplicações Clínicas. International Archives of Otorhinolaryngology. 1997;1;2.
39. Sousa LCA et al..Eletrofisiologia da audição e emissões otoacústicas. 2ª ed. Ribeirão Preto: Novo Conceito. 2010.
40. Frota S. Fundamentos em Fonoaudiologia. Guanabara Koogan. 2003.
41. Fetoni AR, De Bartolo P, Eramo SL, Rolesi R, Paciello F, Bergamini C, Fato R, Paludetti G, Petrosini L, Troiani D. Noise-induced hearing loss (NIHL) as a target of oxidative stress-mediated damage: cochlear and cortical responses after an increase in antioxidant defense. J Neurosci. 2013 Feb 27;33(9):4011-23.
42. Church MW, Hotra JW, Holmes PA, Anumba JI, Jackson DA, Adams BR. Auditory brainstem response (ABR) abnormalities across the life span of rats prenatally exposed to alcohol. Alcohol Clin Exp Res. 2012;6(1):83-96.

43. Church M, Williams H and Holloway J. Brain-stem auditory evoked potentials in the rat: Effects of gender, stimulus characteristics and ethanol sedation. Electroencephalography and Clinical Neurophysiology/Evoked Potentials Section. 1984;59(4), 328–339.
44. DaMatta RA. Modelos Animais na Pesquisa Biomédica. Scientia Medica (Porto Alegre) 2010;20(3):210-211.
45. Reis AD, Dalmolin SP, Dallegrave E. Animal models for hearing evaluations: a literature review. Rev CEFAC. 2017;19(3):417-28.
46. Escabi CD, Frye MD, Trevino M, Lobarinas E. The rat animal model for noise-induced hearing loss. J Acoust Soc Am. 2019;146(5):3692.
47. Ministério da Ciência, Tecnologia e Inovação. 2014. Rede Nacional de Métodos Alternativos (RENAMA). Acesso em: 17 de maio 2020. Disponível em: http://renama.org.br/

4. JUSTIFICATIVA

Considerando o elevado uso de agrotóxicos no Brasil, principalmente de inseticidas – incluindo uso doméstico e em saúde pública, os potenciais danos à saúde causados por essas substâncias isoladas e em associação, bem como os escassos estudos acerca da ototoxicidade secundária à exposição a tais agentes, tornam-se necessários estudos que identifiquem e expliquem possível nexo-causal. Neste sentido, modelos animais cumprem um papel fundamental no delineamento experimental centrado no controle de vieses inerentes à pesquisa em toxicologia com humanos.

5. OBJETIVOS

GERAL

Avaliar os efeitos sistêmicos e auditivos periférico e central da exposição subcrônica inalatória aos agrotóxicos organofosforado Diclorvós e piretróide Cipermetrina, bem como sua associação em ratos Wistar.

ESPECÍFICOS

- Detectar possíveis mudanças na amplitude das EOAPD nas frequências de 4, 6, 8, 10 e 12 kHz e na latência da onda P_{II} no PEATE em ratos Wistar expostos aos agrotóxicos Diclorvós, Cipermetrina e sua associação.
- Avaliar o desenvolvimento ponderal de ratos Wistar durante o período de exposição aos agrotóxicos Diclorvós, Cipermetrina e sua associação.
- Descrever sinais clínicos decorrentes da exposição aos agrotóxicos Diclorvós, Cipermetrina e sua associação em ratos Wistar.
- Avaliar níveis de colinesterase plasmática em ratos Wistar expostos aos agrotóxicos Diclorvós, e sua associação com a Cipermetrina.
- Avaliar a massa relativa dos órgãos expostos aos agrotóxicos Diclorvós, Cipermetrina e sua associação.

6. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

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Original articles

Ototoxicity of an association of insecticides compounds containing dichlorvos and cypermethrin in Wistar rats

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ABSTRACT

Purpose: to evaluate possible ototoxicity secondary to exposure to a combination of pesticides (dichlorvos and cypermethrin based insecticides).

Methods: the Wistar rats were divided into 3 groups (12 animals per group): control (water), positive control for hearing damage (cisplatin) and experimental (exposed to dichlorvos and cypermethrin). The amplitude of distortion product otoacoustic emissions was assessed before and after the exposure. Systemic toxicity signs were also evaluated (clinical signs, weight gain and plasma cholinesterase). Wilcoxon test analyzed the post-exposure amplitudes compared to pre-exposure and Kruskal Wallis following Dunn's post hoc tests analyzed the amplitudes' variation. Normally distributed variables were evaluated by Student's t test.

Results: body weight and plasma cholinesterase values were similar comparing the pre and post exposure in the experimental group. The control group did not manifest significant amplitude reduction of otoacoustic emissions between the pre and post evaluation. In the group exposed to cisplatin there was a significant reduction in amplitudes at 12 kHz on the right ($p = 0.006$; Wilcoxon) and 4 kHz on the left ($p = 0.032$; Wilcoxon). In the group exposed to pesticides, there was a significant reduction in the right ear at 4 kHz ($p = 0.034$; Wilcoxon) and 8 kHz ($p = 0.019$; Wilcoxon) and in the left ear at 4 kHz ($p = 0.007$; Wilcoxon), 6 kHz ($p = 0.023$; Wilcoxon), 8 kHz ($p = 0.045$; Wilcoxon) and 12 kHz ($p = 0.028$; Wilcoxon).

Conclusion: there was ototoxicity in the experimental group, without a relevant systemic toxicity.

Keywords: Ototoxicity; Agrochemicals; Rats; Audiology

INTRODUCTION

Pesticides are commonly used in public health and agriculture[1] and the associations between organophosphates and pyrethroids are widely performed. However, when associated, pesticides can lead to unpredictable effects, considering the complexity of toxicological interactions[2]. In general, pesticides are correlated with more unfavorable outcomes, in relation to neurodevelopment, and their association with sensory functioning has not been completely elucidated[3].

Some studies indicate that exposure to pesticides can cause impairment in hearing[4-6] and in health, in general[7]. Nevertheless, hearing injury is often multifactorial[4,5] and may involve factors such as noise, vibration, use of antibiotics, chemotherapy, and genetic factors. It is difficult to elucidate the isolated cause of hearing damage, considering all the etiologic agents known[8].

Organophosphates (OPs), such as dichlorvos, are pesticides broadly used in agriculture as insecticides. OPs inhibit cholinesterase, causing several intoxications. Pyrethroids are compounds synthetically derived from chrysanthemum that prolong the inflow of sodium and are closely related to hypersensitivity reactions. Cypermethrin is a type II pyrethroid, which has an alpha cyan group and is more potent. Its mechanism of action involves blocking nerve conduction, persistent depolarization, reduction of the action potential amplitude and depolarization in axonal conduction. There is also a suppression of chloride channels related to the GABA receptor[9-12]. In isolation, both substances caused hearing damage in Wistar rats exposed sub-chronically by inhalation. The choice of the sub-chronic route in studies that evaluate the toxicity of pesticides is essential, since this is a frequent route of intoxication in humans and animals associated with numerous cases of chronic or sub-chronic exposure[9,10].

Considering the multifactorial nature of hearing loss, a study conducted in rats - exposed exclusively to pesticides, isolating hearing loss etiologic factors as ototoxic medications or noise - allows to characterize the hearing damage due to exposure to pesticides, as well as its mechanisms. Thus, it is relevant to study exposure to pesticides, widely used, such as organophosphates and pyrethroids, isolated and in combination. Therefore, the present study aims to evaluate the auditory effects of the association of dichlorvos and cypermethrin administered sub-chronically, via inhalation, in Wistar rats.

METHODS

The Federal University of Health Sciences of Porto Alegre Ethics Committee on Animal Use, Brazil, approved the experimental protocol under number 478/16. To reduce the risk of pain or suffering to the animals, the protocol followed the procedures established by the National Council for the Control of Animal Experimentation. The experimental protocol was based on the guidelines for sub-chronic inhalation toxicity test, number 413, proposed by the Organization for Economic Co-operation and Development[13] (OECD) for the Testing of Chemicals.

Chemical agents

An organophosphorus insecticide containing dichlorvos as active ingredient and a pyrethroid insecticide containing cypermethrin as active ingredient were associated. The pesticides were mixed with distilled water (concentration of 1.48mg/L for dichlorvos and at the concentration of 0.25mg/L for cypermethrin). The chosen concentration corresponded to 1/10 of rat inhalation LC50 (LC50 is the concentration that causes the death of 50% of animals exposed to a certain substance).

Cisplatin, an antineoplastic drug, was chosen as a positive control for hearing damage (8mg/kg intraperitoneally, once a day, during 3 days). The drug was mixed with physiological solution (10 mL of solution per kg).

Animal Model

The animals chosen for the experimental protocol were Wistar rats (*Rattus norvegicus*). The animals (36 male rats, aged 60 days, 300 ± 50g) received water and food *ad libitum*, except during exposure, in a cycle of 12h light/dark (controlled conditions of the vivarium).

Experimental

The experimental protocol was based on previous studies[9,10]. The animals included in the experimental protocol had distortion product otoacoustic emissions (DPOAE) present in the frequencies of 4, 6, 8, 10 and 12 kHz. Furthermore, an otoscopy was performed by a veterinarian and no signs of external ear pathology were found in any of the rats. Inhalation exposure chambers acoustically treated by an enclosure (avoiding noise) were coupled to ultrasonic nebulizers as an inlet stream and as an exhaust system an aspirator was coupled

to the chambers. Each chamber had 56 L of volume and accommodated 4 animals following the volume proposed by the OECD[9,13]. To determinate the sample size, a previous research[14] that evaluated rats exposed to cisplatin, an ototoxic substance, was analyzed and considered.

For 5 days (adaptation period) the animals were habituated to the instruments of auditory evaluation and to exposure chamber using only air flow (for 1h on day 1, 2h on day 2, 3h on day 3 and 4h on day 4). Water vapor was used with the air flow on day 5 for 4 hours. The animals (N=36) were allocate at random to three groups: negative control (n=12; animals exposed to inhaled water vapor during 4h, 5 times a week, for 6 weeks), positive control (n=12; animals exposed to 8mg/kg intraperitoneal cisplatin, once a day, during 3 successive days) and experimental (n=12; animals exposed to an inhaled association of the pesticides at the concentration of 1.48mg/L for dichlorvos and at the concentration of 0.25mg/L for cypermethrin, for 4h, 5 times/week, during 6 weeks).

Evaluations

Clinical signs and body weight

On exposure days the rats were assessed for body weight and clinical signs, as tremor, dyspnea, excitation, depression and piloerection.

Distortion product otoacoustic emissions (DPOAEs)

The distortion product otoacoustic emissions were tested (4, 6, 8, 10 and 12 kHz) in the control and experimental groups before (pre-exposure) and after (post-exposure) the exposure period (0 and 42 days), and in the positive control immediately before the 1st administration and 24 hours after the 3rd administration of cisplatin. DPOAEs were performed without anesthetic. DPOAEs were recorded using f1 and f2 tones as acoustic stimuli at 65 and 55db sound pressure level. A child size hearing probe was inserted into the external acoustic meatus of the animal to perform the test. DPOAEs were recorded in the control and experimental groups before (pre-exposure) and after (post-exposure) the exposure period (0 and 42 days), and in the positive control previously the 1st administration and 24 hours after the 3rd administration. The DPOAEs were assessed before and after exposure and the amplitude of response was compared between groups (control, positive control and pesticides).

Euthanasia

Euthanasia was conduct 24 hours post the last exposure to substances (cisplatin, pesticides or water). Intraperitoneal sodium thiopental (40mg/kg) associated with lidocaine (10mg/ml) was previously used as anesthesia. The animals' blood was collected from the caudal vena cava and placed in an EDTA tube, centrifuged and the plasma was frozen and later analyzed to determinate the plasma cholinesterase activity. Organs (spleen, liver, heart, lungs and kidneys) were inspected for macroscopic alterations.

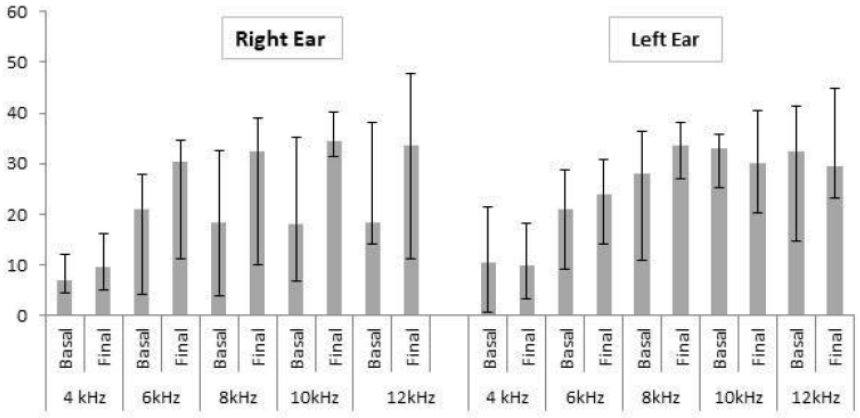
Statistical Analysis

The DPOAE were analyzed by interquartile ranges and median. Wilcoxon test analyzed the post-exposure amplitudes compared to pre-exposure. Kruskal Wallis following Dunn's post hoc tests analyzed the amplitudes' variation (final amplitude minus basal). Parametric data were presented by mean and standard error of the mean. Normally distributed variables, such as cholinesterase activity and body mass, were evaluated by Student's t test. The tests had a 95% confidence interval.

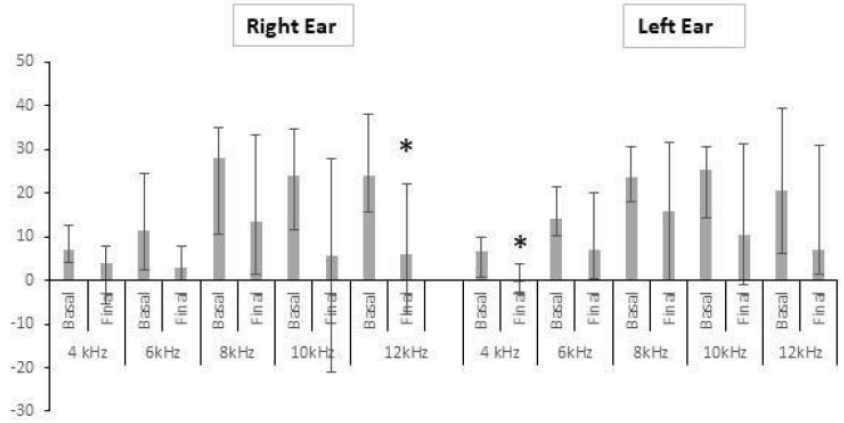
RESULTS

Distortion product otoacoustic emissions

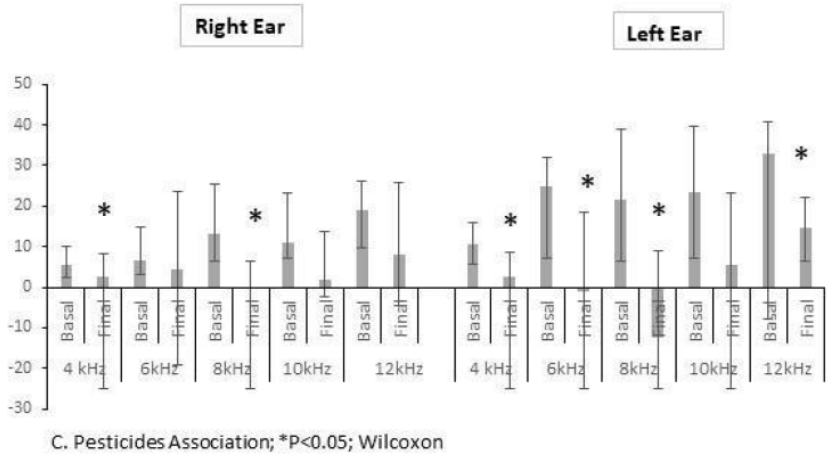
Regarding the pre- and post-exposure amplitudes, the Wilcoxon test (Figure 1) did not demonstrate a significant reduction for the frequencies analyzed in the control group, confirming the robustness of the model. For the same test, in the group exposed to cisplatin there was a significant reduction in amplitudes pre and post exposure at 12 kHz on the right ($p = 0.006$; Wilcoxon) and 4 kHz on the left ($p = 0.032$; Wilcoxon). In the group exposed to pesticides, there was a significant reduction in amplitudes pre and post exposure in the right ear at 4 kHz ($p = 0.034$; Wilcoxon) and 8 kHz ($p = 0.019$; Wilcoxon); in the left ear, there was a significant reduction at 4 kHz ($p = 0.007$; Wilcoxon), at 6 kHz ($p = 0.023$; Wilcoxon), at 8 kHz ($p = 0.045$; Wilcoxon) and at 12 kHz ($p = 0.028$; Wilcoxon).



A. Control; P>0.05; Wilcoxon



B. Positive Control for Hearing Damage (Cisplatin); *P<0.05; Wilcoxon



C. Pesticides Association; *P<0.05; Wilcoxon

Figure 1. Median and interquartile ranges of DPOAE amplitudes pre (basal) and post (final) exposure to pesticides, cisplatin (positive control for hearing damage) and water (control) – Wilcoxon test.

According to the variation in amplitudes (final assessment minus initial assessment - Figure 2), in the association group there was a significant decrease in the frequencies 8 kHz, 10 kHz and 12 kHz on the right and 6 and 8 kHz on the left ear ($p < 0.05$, Kruskal Wallis). Table 1 shows which groups were different from each other in frequencies analyzed according to Dunn's Post Hoc test.

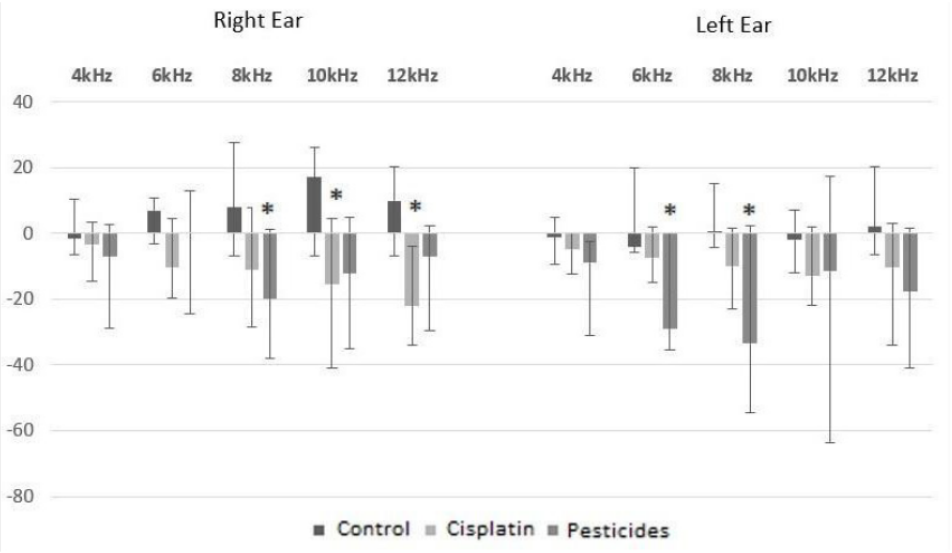


Figure 2. Variation of the amplitudes (medians and interquartile ranges) between the pre and post-exposure period to control (water), positive control (cisplatin) and pesticides.

Table 1. Kruskal-Wallis and Dunn's post hoc tests results

Ear	Frequency	Kruskal-Wallis	Dunn's Post Hoc		
			Control x Pesticides	Control x Cisplatin	Cisplatin x Pesticides
Right	4kHz	p=0.158			
	6kHz	p=0.107			
	8kHz	p=0.014*	p=0.012*		
	10kHz	p=0.024*		p=0.034*	
	12kHz	p=0.011*		p=0.008*	
Left	4kHz	p=0.080			
	6kHz	p=0.049*	p=0.045*		
	8kHz	p=0.009*	p=0.007*		
	10kHz	p=0.370			
	12kHz	p=0.075			

Kruskal-Wallis test; *p<0,05
Dunn's Post Hoc test; *p<0,05

Systemic toxicity signs

No statistically significant change occurred in plasma cholinesterase and body mass gain in the association group. Transient clinical signs (during the exposure period) were observed, including piloerection, reduced activity, dyspnea and pruritus. In euthanasia, there were no differences in the relative mass of the organs or macroscopic changes.

DISCUSSION

The control and positive control groups behaved as expected, once at the control (water) group there was no reduction and the positive control (cisplatin) showed reduction between their final DPOAEs amplitude values compared to the initial one, which corroborates the consistency of the experimental protocol. Thus, the difference (final minus initial) presented by the pesticides' association group indicates that there is an alteration in DPOAEs amplitude values due to sub-chronic inhalation exposure to dichlorvos and cypermethrin association.

Previous studies[9,10] carried out with the active ingredients alone, using the same protocol, resulted in decreased amplitudes in exposure to dichlorvos (8 kHz and 10 kHz bilaterally) and cypermethrin (8, 10 and 12 kHz bilaterally and 4 and 6 kHz on the right). In the present study, the association of both was able to decrease the amplitudes of DPOAEs between pre and post exposure at 4 kHz and 8 kHz on the right and 4, 6, 8 and 12 kHz on the left.

The similarity between the amplitude variations (final minus initial) between the association group and the cisplatin group ($p < 0.05$, Kruskal Wallis) also indicates that the pesticides tested in association are comparable to the ototoxic drug (positive control). Regarding this variation, the decrease in the association group compared to the control at 8 kHz on the right and 6 and 8 kHz on the left ear ($p < 0.05$, Kruskal Wallis; $p < 0.05$, Dunn's post hoc), and its similarity with the positive control group is congruent with previous studies that evaluated DPOAEs before and after exposure to dichlorvos[9] and cypermethrin[10] alone in Wistar rats. The exposure to cypermethrin generated a reduction in the amplitude variation in 10 and 12 kHz on the right and in 8, 10 and 12 kHz on the left. Dichlorvos caused a reduction in the amplitude variation in 10 kHz on the right and 8kHz and 10 kHz on the left. Such results indicate hearing damage secondary to exposure to ototoxic agents, since they occur at high frequencies and were pointed out after assessment of DPOAEs, an assessment sensitive to cochlea's hair cells damage.

Regarding the ototoxic results manifested without significant alterations in the clinical signs, as well as no reduction in the plasmatic cholinesterase activity, representing absence of systemic toxicity, these findings are similar to the ones evidenced in previous studies using the same model[9,10]. The concentrations of pesticides in those studies, as well as in the present study, corresponded to 1/10 of the inhaled LC50 for rats, which represents that even at low concentrations there was damage to the DPOAEs, without clinical signs indicative of toxicity or even changes in plasma cholinesterase. A normal range of plasma cholinesterase levels was also found in workers chronically exposed to organophosphates, suggesting relative resistance of higher nervous system functions to mild chronic pesticide exposure[15].

Although studies in humans have already suggested the correlation between hearing damage and exposure to pesticides[16,17], most research have limitations, once human beings may be exposed to more than a factor that contributes to this outcome, such as noise, vibration and use of ototoxic drugs. The exact mechanisms of cochlear function damage secondary to exposure to pesticides are not yet well established, however, similar changes induced by medications indicate that oxidative stress might play a role in this issue and in toxicity in humans and animals in general[18,19]. The sensorineural hearing loss mechanism associated with cisplatin and aminoglycosides occurs due to damage to the outer hair cells, and specific aminoglycosides, such as gentamicin, can still damage the vestibular system. In this case, oxidative stress would occur in the inner ear followed by cell damage[20]. Lee *et al.* (2016)[21], in research that evaluated the effect of a catalase inhibitor as a protector against ototoxicity in mice, found out that apparently the ototoxic effects of cobalt would be related to the generation of reactive oxygen species (ROS) and the production, in the auditory system, of pro-inflammatory cytokines. Still, Jiang *et al.* (2016) also report, in their research that investigated ototoxicity by inducing cell death by aminoglycoside, the contribution of the generation of ROS in ototoxicity[22].

Kim *et al.* (2017)[23] evaluated the role of the autophagosome marker LC3-II, related to the induction of damage in vivo and in vitro. These authors suggest that autophagic flux is a possible mechanism for gentamicin-induced ototoxicity. In this case, the damage could be related to the accumulation of autophagosomes, due to impaired autophagy. Choi *et al.* (2019)[24]

investigated the relationship between cisplatin-induced hearing loss and receptor-interacting protein kinase (RIP) 3-dependent necroptosis in vivo and in vitro, concluding that RIP3-dependent necroptosis was highly expressed in cisplatin-induced ototoxicity. The authors noted that the Corti organ and the spiral ganglion neurons were sites particularly susceptible to the process of necroptosis.

There may be some potential limitations in this study. Although hearing damage caused by pesticides may be similar to the ones caused by other ototoxic substances (sensory-neural and damaging to the hair cells of the cochlea, frequently causing a decrease in the amplitude of otoacoustic emissions), the lack of previous studies analyzing specifically the mechanisms that cause such damage after exposure to pesticides make this aspect unclear. Further studies need to be conducted in order to clarify the decrease in cochlear function related specifically to this type of exposure. Such studies could associate electrophysiological results with morphological data of structures such as hair cells, which could better elucidate the mechanism of hearing injury by pesticides. Understanding these mechanisms is a major matter for the development of studies that evaluates substances with otoprotective activity.

CONCLUSION

Considering the ototoxicity secondary to sub-chronic inhalation exposure to the association of compounds containing cypermethrin and dichlorvos, even without relevant systemic toxicity, the importance of raising awareness of individuals occupationally exposed to pesticides, even at low concentrations, about the importance of performing their work activities with the use of personal protective equipment, is highlighted. Furthermore, measures seem to be important to regulate audiometric control in workers exposed to pesticides and not only in those exposed to noise, since strong evidence points to the risk of ototoxicity secondary to exposure to this type of substance.

ACKNOWLEDGMENTS

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REFERENCES

1. Zeigelboim BS, Malisky JS, Rosa MR, Lacerda ABM, Alcaraz PS, Fonseca VR. The importance of otoneurological evaluation in Brazilian workers exposed to pesticides: a preliminary study. Int. Arch. Otorhinolaryngol. 2019;23(4):389-95.
2. Hernández AF, Gil F, Lacasaña M. Toxicological interactions of pesticide mixtures: an update. Arch Toxicol. 2017;91(10):3211-23.
3. Sturza J, Silver MK, Xu L, Li M, Mai X, Xia Y et al. Prenatal exposure to multiple pesticides is associated with auditory brainstem response at 9months in a cohort study of Chinese infants. Environ Int. 2016;92-93:478-85.
4. Kós MI, Hoshino AC, Asmus CIF, Mendonça R, Meyer A. Efeitos da exposição a agrotóxicos sobre o sistema auditivo periférico e central: uma revisão sistemática. Cad Saúde Pública. 2013;29(8):1491-1506.
5. Alcarás PAS, Larcerda ABM, Marques JM. Study of Evoked Otoacoustic Emissions and suppression effect on workers exposed to pesticides and noise. CoDAS. 2013;25(6):527-33.
6. Teixeira CF, Augusto LGS, Morata TC. Saúde auditiva de trabalhadores expostos a ruído e inseticidas. Rev Saúde Pública. 2003;37(4):417-23.
7. Cazé AMB, Lacerda ABM, Lüders D, Conto J, Marques J, Leroux T. Perception of the quality of life of tobacco growers exposed to pesticides: emphasis on health, hearing, and working conditions. Int. Arch. Otorhinolaryngol. 2019;23(1):50-9.
8. Reis AD, Dalmolin SP, Dallegrave E. Animal models for hearing evaluations: a literature review. Rev. CEFAC. 2017;19(3):417-28.
9. Reis AD, Cunha EO, CostaValle MT, Machado MS, Dallegrave E. Effects of subchronic inhalation exposure to an organophosphorus insecticide compound containing dichlorvos on wistar rats' otoacoustic emissions. Braz J Otorhinolaryngol. 2022;88(1):28-35.
10. Cunha EO, Reis AD, Macedo MB, Machado MS, Dallegrave E. Ototoxicity of cypermethrin in Wistar rats. Braz J Otorhinolaryngol. 2019;86(5):587-92.
11. Filho ADA, Souza SD. Anticolinesterásicos. In: Filho ADA, Campolina D, Dias MB, editors. Toxicologia na prática clínica. 2nd ed. Belo Horizonte: Folium; 2013. p. 89-95.

12. Wakeling EN, Neal AP, Atchison WD. Pyrethroids and their effects on ion channels. In: Soundararajan R, editor. Pesticides - advances in chemical and botanical pesticides [Internet]. London: IntechOpen. 2012. Available from: https://www.intechopen.com/chapters/37969 doi: 10.5772/50330.
13. OECD. Test No. 413: subchronic inhalation toxicity: 90-day study, OECD guidelines for the testing of chemicals, Section 4. 2009. OECD Publishing, Paris.
14. De Freitas MR, Figueiredo AA, Brito GAC, Leitão RFC, Junior JVC, Junior RMG et al. The role of apoptosis in cisplatin-induced ototoxicity in rats. Braz J Otorhinolaryngol. 2009;75(5):745-52.
15. Rodnitzky RL, Levin HS, Mick DL. Occupational exposure to organophosphate pesticides. Archives of Environmental Health: An International Journal. 1975;30:2:98-103.
16. Sena TRR, Dourado SSF, Lima LV, Antoniolli ÂR. The hearing of rural workers exposed to noise and pesticides. Noise Health. 2018;20(92):23-6.
17. Tomiazzi JS, Pereira DR, Judai MA, Antunes PA, Favareto APA. Performance of machine-learning algorithms to pattern recognition and classification of hearing impairment in Brazilian farmers exposed to pesticide and/or cigarette smoke. Environ Sci Pollut Res Int. 2019;26(7):6481-91.
18. Medithi S, Jonnalagadda PR, Jee B. Predominant role of antioxidants in ameliorating the oxidative stress induced by pesticides. Arch Environ Occup Health. 2021;76(2):61-74.
19. How V, Hashim Z, Ismail P, Omar D, Said SMD, Tamrin SBM. Characterization of risk factors for DNA damage among paddy farm worker exposed to mixtures of organophosphates. Arch Environ Occup Health. 2015;70(2):102-90.
20. Schacht J, Talaska AE, Rybak LP. Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. Anat Rec (Hoboken). 2012;295(11):1837-50.
21. Lee JN, Kim SG, Lim JY, Dutta RK, Kim SJ, Choe SK et al. 3-Aminotriazole protects from CoCl2-induced ototoxicity by inhibiting the generation of reactive oxygen species and proinflammatory cytokines in mice. Arch Toxicol. 2016;90(4):781-91.
22. Jiang P, Ray A, Rybak LP, Brenner MJ. Role of STAT1 and oxidative stress in gentamicin-induced hair cell death in organ of corti. Otol Neurotol. 2016;37(9):1449-56.
23. Kim YJ, Tian C, Kim J, Shin B, Choo OS, Kim YS et al. Autophagic flux, a possible mechanism for delayed gentamicin-induced ototoxicity. Sci Rep. 2017;7:41356.
24. Choi MJ, Kang H, Lee YY, Choo OS, Jang JH, Park SH et al. Cisplatin-induced ototoxicity in rats is driven by RIP3-dependent necroptosis. Cells. 2019;8(5):409.

7. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

"Ototoxicity of Insecticides in Wistar Rats' Auditory Parameters"

Aléxia dos Reis, Charles Francisco Ferreira, Mateus Belmonte Macedo, Márcia Salgado Machado, Eliane Dallegrave

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ABSTRACT

Purpose: The objective of this research was to evaluate the effects of an organophosphate (OP) and a pyrethroid (PYR) on the hearing function of rats by distortion product otoacoustic emissions (DPOAEs) and brainstem evoked response audiometry (BERA) pre and post 6 weeks of exposure. Methods: Rats were divided into five groups (5 animals/group): negative control (water), positive control (cisplatin), OP, PYR, and association (OP + PYR). Results: There were no alterations in clinical signs nor body mass parameters. The cisplatin group presented lower DPOAEs medians post exposure in 4kHz, 10kHz and 12kHz. The PYR group had lower amplitudes in 6kHz and 8kHz and the OP + PYR group showed lower amplitudes in 12kHz. Both the cisplatin group and the OP group presented higher median after the exposure period. At BERA test on both ears, the cisplatin group presented a higher median when compared to the water group. On the left, the OP + PYR group had lower median in relation to the cisplatin group. Bilaterally the OP + PYR group presented similar means to the water group. Conclusion: There was secondary ototoxicity to the exposure of the evaluated pesticides, without relevant systemic intoxication.

Keywords: pesticides; toxicology; rats; audiology.

MAIN TEXT

Title: Ototoxicity of Insecticides Evaluated by BERA and DPOAE Parameters.

INTRODUCTION

Use of pesticide reduces qualitative and quantitative losses in agriculture, which maintains the economic sustainability of the producer [1], increases production efficiency, and reduces the cost of food [2]. It is also important to highlight the role of domestic insecticides, often composed of pyrethroids, in combating vectors [3] such as *Aedes aegypti*, which spreads diseases such as dengue, yellow fever, zika fever and *chikungunya*. However, such substances represent a significant risk to the environment and human health, being the subject of current research[4]. Exposure to pesticides is correlated with unfavorable outcomes in neurodevelopment, but their effects on the body's sensory functioning have not been fully elucidated [5].

Among the widely used classes of pesticides are organophosphate (OP) and pyrethroid (PYR) insecticides. The mechanism of action of OPs involves the irreversible inhibition of the enzyme acetylcholinesterase (AChE), whose function is to hydrolyze the neurotransmitter acetylcholine (ACh), resulting in cholinergic effects (e.g., salivation, muscle weakness, changes in mental status). In addition, OPs can induce oxidative stress imbalance, axonal transport deficits, neuroinflammation and autoimmunity reactions [6]. On the other hand, PYR are sodium channel modulators, and in PYR type I, the action is primarily mediated by voltage-dependent sodium channels, while in PYR type II, they also act on voltage-dependent chloride channels. Type II PYR bind to

inhibitory gamma-aminobutyric acid (GABA) receptors in the central nervous system (CNS), blocking chloride channels and their activation. With the absence of synaptic inhibition caused by GABA, hyperexcitability occurs. Thus, both type I and II PYR prolong the depolarization time, generating hyperexcitability [7,8] in target and nontarget species [9]. The toxic effects of PYR often include irritation of the skin, airway, and hypersensitivity (e.g., anaphylactic reactions such as bronchospasm, oropharyngeal edema, and shock) [10].

Research conducted in animal models showed ototoxicity in *Wistar* rats exposed to type II PYR cypermethrin and the OP dichlorvos, as well as the occurrence of ototoxicity when both substances were combined [11,12]. OP and PYR insecticides can be associated in commercial formulations or even applied to the same crops, resulting in concomitant exposure, which can trigger antagonistic, additive, or synergistic neurotoxic effects [13]. The combination of pesticides is frequent in developed or developing countries. There is a likely association between exposure to mixtures of pesticides and higher rates of morbidity and lethality [14], which may be related to the unpredictable effects of the combination of compounds, considering the complexity of their toxicological interactions [15]. This scenario justifies the importance of analyzing the effect of inhaling these combined substances, which is often how people are exposed to these and other pesticides while at work [16].

Health effects caused by pesticides has already been well explored by previous research. However, ototoxicity secondary to pesticide exposure needs further attention because there are few studies on the subject. Auditory function involves the integration of several structures, being evaluated through specific

tests. In addition, the use of animal models in hearing damage research avoids confounding factors, such as genetics, presbycusis, use of ototoxic drugs, previous diseases, and environmental circumstances (e.g., noise, vibration), which can contribute to hearing damage. Previous studies identified alterations in auditory parameters based on distortion product otoacoustic emissions when OP and PYR were analyzed separately. However, the effects of its association are not yet clear, especially at the level of the CNS. Therefore, the present study aimed to evaluate the effects of dichlorvos (an OP), and cypermethrin (a PYR), and their combination (OP+PYR), on the hearing function of *Wistar* rats through the examination of distortion product otoacoustic emissions (DPOAEs) and the brainstem evoked response audiometry (BERA).

METHODS

CHEMICAL AGENTS

The OP + PYR group was exposed to commercial formulations containing dichlorvos (OP) and cypermethrin (PYR) as active ingredients. Both substances were diluted in distilled water at the following concentrations: 1.48mg/L for dichlorvos and 0.25mg/L for cypermethrin, corresponding to 1/10 of rat inhalation median lethal concentration 50 (LC50). The same concentrations (1/10 of rat inhalation LC50) were used in the groups exposed to dichlorvos and cypermethrin alone. Cisplatin, a chemotherapeutic agent known by its ototoxicity, was chosen as a positive control for hearing damage. It was diluted in physiological solution (10mL of NaCl 0,9%/kg) and the animals were treated with 8mg/kg i.p. once daily, for 3 consecutive days [11,12,17].

EXPERIMENTAL ANIMALS

Male Wistar rats (N=25, 5 animals per group), were 60 days old and weighed 300±50g at the beginning of this study. The animals were kept under controlled conditions of the bioterium, 12h light/dark cycle, receiving water and food *ad libitum*, except during exposure period.

EXPERIMENTAL PROTOCOL

This research was approved by the XXXXXXXXXXXXX Ethics Committee on Animal Use Ethics Committee on Animal Use (CEUA) (n°XXXX). To ensure the welfare of the animals and avoid pain or distress, all the procedures of health care (according to the National Council for the Control of Animal Experimentation – CONCEA) were followed. The ARRIVE guidelines were used to guarantee the data reliability.

Inclusion criteria were no signs of external ear pathology (verified by otoscopy performed by a veterinarian), Distortion Product Otoacoustic Emissions (DPOAEs) present in all tested frequencies (4, 6, 8, 10 and 12kHz) and p2 wave latency (BERA) within normal values prior to the beginning of the experimental period. Animals that presented otoscopy alterations, or absence of any of the tested frequencies in DPOAEs, or abnormal values at p2 wave latency (BERA) were excluded of the protocol.

This experimental protocol was adapted from the subchronic inhalation toxicity test (repeated exposure - number 413), from the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals [18].

During the exposure period (subchronic inhalation exposure), the rats were kept in soundproof inhalation exposure chambers. The chambers had 56L of volume and were coupled to ultrasonic nebulizers as an inlet stream, and to an aspirator as an exhaust system. Following the volume proposed by the OECD, each chamber accommodated 5 animals.

The animals were allocated randomly into the groups one by one using aleatory number scale. The rats (N=25) were randomly divided into five groups of five animals each: negative control, positive control for hearing damage (cisplatin), OP insecticide, PYR insecticide and association between OP and PYR. The negative control group was exposed to water (vehicle for dilution of the formulation) by inhalation for 4h, 5 days/week, during 6 weeks. The positive control group was treated with 8mg/kg of cisplatin i.p., once daily, for 3 consecutive days (totaling 24mg/kg). The OP + PYR group was exposed to inhaled dichlorvos (concentration of 1.48mg/L) and cypermethrin (concentration of 0.25mg/L) for 4h, 5 days/week, for 6 weeks. The same concentrations of OP and PYR were used in the groups exposed to the pesticides alone (dichlorvos group and cypermethrin group, exposed to the pesticides for 4h, 5 days/week, during 6 weeks).

ASSESSMENTS

Clinical signs and body mass

Once a week during the exposure period, the rats were weighed (weight in grams) and daily for clinical signs (e.g., depression or excitation, tremor, piloerection, dyspnea) by veterinary evaluation.

Auditory parameters assessments

Auditory parameters were measured using DPOAEs, followed by BERA under anesthesia with Ketamine (50mg/kg) and Xylazine (10mg/kg) i.p. Figure 1 shows a schematic representation of the auditory assessment by distortion product otoacoustic emissions - DPOAEs (A) and brainstem evoked response audiometry – BERA (B). The evaluator who performed the tests did not know which group the animal belonged.

Insert Figure 1

Distortion Product Otoacoustic Emissions (DPOAEs)

DPOAEs test were performed in the frequencies of 4, 6, 8, 10 and 12kHz in all groups with an infant size hearing probe placed into the external ear canal of the animal. Control and experimental groups (OP, PYR and OP + PYR) were evaluated before (pre) and after (post) the exposure period (0 and 42 days). The positive control for hearing damage was assessed immediately before the 1st administration and 24h after the 3rd administration of cisplatin. DPOAEs were recorded using two tones (f1 and f2) as acoustic stimuli (f1/f2, f2:f1 ratio fixed at 1.22) at 65 and 55db sound pressure level (SPL).

Brainstem Evoked Response Audiometry (BERA)

In BERA, a single-channel Smart EP (IHS®) device was used to assess brainstem evoked response audiometry (BERA). The animals were evaluated using three disposable electrodes fixed with microporous tape in the region corresponding to the frontal bone, right and left ear pinna. Insert

earphones with small ear tips were inserted in the animals' external auditory canal. A 70dBNA stimulus was conducted logging the response both in the rarefied and condensed polarity, and each of these records was presented twice to verify the replicability of the results. Every electrophysiologic response needs to be confirmed because only neural responses remain after the stimulus. In case of artifacts the response wears off and for that reason is not true. Therefore, each response was carried out twice to verify if the stimulus remained present, which is called replicability in electrophysiology.

EUTHANASIA

Euthanasia was performed using sodium thiopental overdose (120mg/kg) i.p. 24h after the last inhalation exposure or treatment with cisplatin.

STATISTICS

Normality of the data were determined using the Shapiro-Wilk test, and comparisons between the groups were performed by Kruskal-Wallis test, Wilcoxon test (pre versus post- analysis), or by generalized estimating equations (GEE) model with Bonferroni corrections (used to simultaneously evaluate parameters over time and between groups). For variables with symmetric distribution, the linear model was applied, and for those with asymmetric distribution, the gamma model.

Other symmetric data were presented as mean and standard error of the mean (±SEM) and were evaluated by One-Way Analysis of Variance (ANOVA).

The significance level was set at 5% for all analyses, performed in SPSS, version 18.0 (SPSS version 18.0 for Windows, SPSS Inc., Chicago, IL, USA).

The sample size was calculated based on a previous study that used auditory brainstem responses and distortion product otoacoustic emissions tests to assess auditory function [19].

A Cohen's D test was performed to evaluate the effect size in each group for both auditory tests (DPOAEs and BERA).

The evaluator who performed all the statistical analysis did not know the groups' code.

RESULTS

Clinical signs and body mass

Once cisplatin was diluted in physiological solution and i.p. administered daily as a positive control for ototoxicity (differs from the OP and the PYR groups, administered by inhalation), body mass was not evaluated in this group. Mean (±SEM) pre-exposure body mass of the negative control (water) (387.6±6.43), the OP (360.8±14.21), the PYR (364.8±10.67) and the OP + PYR (373.0±7.01) groups were similar (One-Way ANOVA, $F(3,16)=1.381$, $p=0.284$). Similarly, mean (±SEM) post-exposure body mass of the negative control (water) (427.6±11.02), the OP (420.8±12.70), the PYR (432.8±12.36) and the OP + PYR (448.6±11.97) groups were similar (One-Way ANOVA, $F(3,16)=0.968$, $p=0.432$).

Taken together, during the period of exposure to pesticides, the animals exposed to the OP, to the PYR, and to the OP + PYR groups did not show

altered clinical signs (only in the first 30 minutes of the day one and day two of exposure) or body mass parameters, indicating no relevant systemic toxicity. However, in the assessment of auditory function, ototoxicity was observed.

Auditory parameters

Table 1 shows the DPOAEs amplitudes presented as median (IQR). After exposure period, the positive control (cisplatin) group presented lower amplitude in relation to the pre-exposure, in 4kHz, 10kHz and 12kHz on the right side (Wilcoxon Signed Rank test, p=0.043, p=0.043 and p=0.042, respectively). Besides, the PYR group presented lower amplitude in 6kHz and 8kHz on the left side (Wilcoxon Signed Rank test, p=0.043 for both), and the OP + PYR group showed lower amplitude in 12kHz on the right side (Wilcoxon Signed Rank test, p=0.043).

Insert Table 1

Table 2 shows the BERA latency presented as median (IQR). Both the positive control (cisplatin) and the OP groups presented higher latency at post-exposure period in relation to the pre-exposure (Wilcoxon Signed Rank test; cisplatin p=0.042 on the right and p=0.043 on the left; OP p=0.043 on the right). At post-exposure measurement, in both ears, the positive control group (cisplatin) presented higher latency in relation to the negative control group (water) (Kruskal Wallis, Dunn *post hoc*; right ear p=0.007 and left ear p=0.003). On the left ear, the positive control (cisplatin) group presented higher latency in relation to the OP + PYR group (Kruskal-Wallis, with Dunn *post hoc*; p=0.003).

Insert Table 2

The Cohen's d test evaluated between pre- and post-exposure in each group for both auditory tests (DPOAEs and BERA) demonstrated in the negative control group minimum effect size. It was possible to obtain a large effect size in the positive control group, characterizing the robustness of the chosen model of study with an n=5 animals/group (Table 1 and Table 2).

GEE statistical analysis was used to assess interactions between time and group effects for both auditory tests.

DPOAEs amplitude means (±SEM) of each measured frequency (4kHz, 6kHz, 8kHz, 10kHz, 12kHz), by group and time (pre- and post-exposure period) are displayed in Table 3. On the right side (Table 3 A), a group effect was observed in 4kHz, since the positive control (cisplatin) group presented lower mean (±SEM) in relation to the OP and OP + PYR groups (GEE, p=0.018) and a time effect was observed, since the post mean was lower to pre-exposure (GEE, p=0.005). Also, in 12kHz the positive control (cisplatin) group presented lower mean (±SEM) in relation to the PYR group (GEE, p=0.025).

On the left side (Table 3 B), an interaction was observed in 6kHz, since the PYR group presented lower post mean (±SEM) in relation to the pre-exposure (GEE, p=0.018). Also, in 8kHz an interaction was observed, since post mean (±SEM) was lower than pre-exposure in negative control and PYR groups (GEE, 0.0001). In the 12kHz the interaction was observed, since post mean (±SEM) was lower than pre-exposure for both groups, negative control and PYR (GEE, 0.002). Differences among groups in the pre-exposure were not highlighted for

not presenting relevant clinical differences (in 8, 10 in the left side and 12kHz in both sides).

Insert Table 3.

BERA latency means (±SEM) at the groups and times (pre- and post-exposure period) are displayed in Figure 2 (A. right, B. left). In the right side, a time effect was observed on the positive control (cisplatin) and OP groups, since latency to increase in the post-exposure period in relation to the pre-exposure (GEE, $p \leq 0.05$ for all analysis). A group effect was observed in both ears, since the OP group presented higher means (±SEM) in both ears, in relation to the negative control (water) group (GEE, $p \leq 0.05$ for all analysis). In addition, the PYR group presented higher means (±SEM), in the right ear, in relation to the negative control (water) group (GEE, $p \leq 0.05$ for all analysis). Again, in both ears, the OP + PYR group presented similar means (±SEM) to the negative control (water) group (GEE, $p > 0.05$ for all analysis).

Insert Figure 2.

DISCUSSION

In the present study, we demonstrated that a subchronic inhaled exposure to dichlorvos (organophosphate insecticide, OP), cypermethrin (pyrethroid insecticide, PYR), or to the positive control (cisplatin) was able to change auditory parameters of Wistar rats evaluated by the DPOAEs and the BERA tests, showing ototoxic effects at the cochlea. The authors also highlight that the OP + PYR group presented similar behavior to the negative control

(water) group, and those animals did not show clinical signs or changes in body mass during the study.

Otoacoustic emissions (OAEs) are responses generated by the cochlea in the form of acoustic energy [20]. Its presence indicates normality in relation to the sound response of the cochlear preneural receptor mechanism, as well as the middle ear mechanism [21].

The use of OAEs also allow for the assessment of cochlear mechanical function, obtaining objective information about specific micromechanical activities for preneural or sensory elements of the organ of Corti, cochlear components that are distal to the nerve fiber endings. Clinically and in research, the OAEs monitoring is extremely important since many peripheral auditory dysfunctions (e.g., noise-induced hearing loss, chemical-induced ototoxicity, hereditary hearing loss) originate in sensory components of the cochlea [20]. In addition, BERA originate in the cochlea, auditory nerve and auditory pathways of the brainstem and can be measured relatively easily in humans. In rats, however, it is believed that the BERA has four components (waves I, II, III and IV), that occur within 6ms after the stimulus and reflect the normal activity of the following structures: the auditory nerve (wave I), the cochlear nucleus (wave II), the superior olivary complex (wave III) and the lateral lemniscus (wave IV) [22-24] In rodents, since the BERA's P2 is the largest wave and the last one to disappear with decreasing stimulus, the P2 is often used to derive the rat's latency-intensity curve [22,25,26].

The evaluation in preclinical animal model research allows the control of common biases related to the research of pesticide-induced effects in humans (e.g., genetics, presbycusis, use of ototoxic drugs, previous diseases,

environmental circumstances - noise, vibration), facilitating the investigation of the causal nexus [11,12]. Mammals, in particular, have several advantages in studies to assess hearing functioning (e.g., anatomical characteristics similar to those of humans) [27]. Rodents are the most used models, and the rat presents anatomical and physiological similarity with humans, being an excellent model for health studies (e.g., good adaptability, robustness, good ability to survive and intelligence) [27,28].

As mentioned before, authors showed in previous studies the ototoxic effects of these pesticides, in isolation, at the cochlear level. On the other hand, as far as the authors know, this is the first study in which the association of OP + PYR was tested for ototoxicity by the BERA examination, a brainstein hearing functional assessment. The individual behavior of each chemical was characterized by interferences in different regions of the auditory system, with the outer hair cells being more affected by the PYR and the cochlear nucleus by the OP. The cochlear ototoxicity mechanism can be related to the capacity of PYR and the OP to blocking block voltagegated channels in response to their effects on specific receptors (e.g., sodium, chloride and calcium). By these effects, several muscles enrolled in the middleear protective reflex are mediated by acetylcholine release (e.g., stapedius muscle) [29,30]. Preclinical and clinical studies indicate that pre- and post-natal exposure to OPs may affect numerous brain regions, and that the behavioral consequences of OP exposure depend on the level as well as the timing of exposure [31,32]. In contrast to OPs, the PYR exposure effects are still poorly understood.

There is similarity in hearing loss secondary to exposure to industrial chemical agents, ototoxic drugs (e.g., aminoglycosides and cisplatin, our

positive control) and the ones caused by noise. These effects show a tendency to be irreversible at higher frequencies, symmetrical, bilateral, and predominantly damage the cochlear hair cells [33].

Systemic synergistic effects have been reported when OP and PYR are combined, as OPs inhibit esterases, which reduces PYR hydrolysis [7,34]. However, such synergism did not occur in the ototoxicity observed in this study, since when evaluating the association of active ingredients (the OP + PYR group), the effects became smaller than those evidenced for each of the isolated ingredients (the OP and the PYR groups), regardless of the region evaluated. Our data demonstrates that the PYR acted on the cochlear functions, since there was a DPOAEs amplitude reduction. The OP affected the auditory nerve fiber to a greater extent, considering that there was a greater P2 latency in the BERA test.

In the positive control (cisplatin) group, the DPOAEs and the BERA tests were different between the pre and the post-exposure times, bilaterally, as expected. These results confirmed the robustness of the chosen model, considering the already established ototoxicity caused by the cisplatin. In addition, there was a significant reduction in the hearing functioning displayed by the OP group, since in both tests, paired analysis identified a higher latency during post-exposure period, in relation to the pre-exposure.

Surprisingly, the OP + PYR group did not differ from the negative control (water) group in hearing assessments. Many pesticides, including OPs and PYRs, can modify the action of the efferent auditory system by cholinergic modulation, causing direct/indirect effects in the sensory pathways and the peripheral auditory system. Damage to vestibular and auditory systems are

associated with speech perception difficulties, dizziness, and tinnitus. Cypermethrin, a class II PYR, prolongs the opening of sodium channel (e.g., antagonizes GABA, leads to hyper-excitation of the CNS, responsible for its neurotoxic effects in mammals). Nevertheless, secondary targets have also been identified in voltage-gated channels (e.g., chloride, calcium, potassium), neurotransmitters and neuromodulators (e.g., glutamate, acetylcholine, ATP, GABA), as well as DNA damage and oxidative stress imbalance in the neuronal cells.

GABAergic modulation by PYR and indirect parasympathomimetic action of OP, may have been why the OP + PYR group displayed a similar hearing function to the negative control (water) group. Although the OP + PYR group did not present audiological damage at the analyzed moment, individual effects of the studied pesticides were observed. This scenario highlights the importance of studies in this field of science, since the auditory effects caused by the simultaneous use of various pesticides are strongly recommended. In addition, our results show peripheral and central damage when each pesticide, alone, is administered. Our data indicates that complementary objective auditory tests, such as otoacoustic emissions and BERA, are necessary when assessing pesticide-exposed subjects besides the tests normally used in the routine auditory assessments. Objective tests are a good alternative to the pure tone audiometry (PTA), a subjective behavioral test commonly used to assess agricultural workers hearing. The PTA test may not show hearing damage once it does not identify minor alterations at the level of outer hair cells (evidenced by DPOAEs) and auditory nerve/cochlear nucleus (evidenced by BERA). The

combined assessment by DPOAEs and BERA tests allows visualizing changes at the cochlear and central level before their appearance in conventional PTA.

Our data agree with a cohort study [5,35] in which the BERA test demonstrated slower latencies in children who had higher prenatal exposure to pesticides. Research indicates that such factors contribute to impaired neuromaturation [5]. Silver *et al.* (2019) also identified slower auditory signal transmission in children exposed to an herbicide, who were evaluated by the BERA test, stressing that the findings can cause delay in auditory development, since latencies reflect the maturation of the auditory pathway [36]. However, the change in the BERA parameters after exposure to pesticides is not a consensus in the literature. A study that evaluated prenatal exposure to the OP through the BERA test, in children aged 6 weeks, 9 and 18 months, found no association between exposure to the pesticide and the speed of signal transmission. In addition, Körbes (2009) did not find changes in the BERA parameters after seven-day exposure of guinea pigs to methamidophos, although the time-window of exposure to the studied pesticides was short [37].

The effects of chronic exposures are difficult to diagnose because of underreporting and nonreporting in the exposed population [38]. The low demand for health services might be explained by the fact that the symptoms are chronic, and the workers adapt to the change in health. They may also be afraid to admit that that the available personal protective equipment (PPE) wasn’t adequate at protecting them. Workers might also be afraid to report changes in health because of a fear of reprisal from the local agribusiness. A causal link between poisoning secondary to exposure to pesticides has been established and reporting symptoms might lead to inspection of work sites [38].

Considering the current Brazilian legislation, the Regulatory Norm 7 (NR7) establishes as mandatory the elaboration and implementation of employers and institutions that admit workers as employees to the Medical Control and Occupational Health Program (PCMSO) [39]. The PCMSO seeks to prevent, track, and improve early diagnosis of health problems related to the occupational activity [38]. According to the NR7 [39] workers exposed to noise must perform audiometric control with reference and sequential exams. The standard does not include workers exposed to chemical agents, such as pesticides. Furthermore, workers exposed to OP and carbamate pesticides should undergo a blood test to determine cholinesterase activity for toxicity assessment [39]. On the other hand, another study, carried out in *Wistar* rats subchronically exposed by inhalation to the OP showed that, although there was ototoxicity, there was no variation in the plasma cholinesterase activity [11]. This indicates that hearing damage can occur, even without changes in cholinesterase activity.

Our study had some limitations (e.g., no neurochemical marker measurement). After extensive research we did not find any explanations about the specific mechanism involving the differences found between the right and the left ears. Nevertheless, new studies might demonstrate clearer why the substances affected one side and not the other in specific frequencies. We present as a positive aspect the route of exposure to the tested agents, mimicking the scenario found in cases of human exposure. It was possible to identify and to confirm functional changes in the audiometric assessments performed. Furthermore, the application of animal models enables the control of interfering variables (e.g., noise), a common bias in clinical studies. It is

possible that more severe changes in auditory function might occur with longer exposures (chronic). In conclusion there was ototoxicity secondary to the exposure of the evaluated pesticides (OP or PYR), without relevant systemic toxicity. PYR acted predominantly on cochlear function, by reducing the DPOAEs amplitude, while the OP affected the nerve fibers of the auditory nerve, by increasing P2 latency in the BERA test. When both, the OP and the PYR pesticides, were combined, the association effects became smaller than those of each of the isolated chemical exposures, regardless of the region evaluated. Although the OP + PYR group did not present audiological damage at the assessment, individual effects of the studied pesticides were observed. This scenario highlights the necessity in applying complementary auditory tests when assessing pesticide-exposed subjects, in addition to the tests normally used in the routine auditory assessments.

REFERENCES:

1. Lopes CA (2017) É possível produzir alimentos para o Brasil sem agrotóxicos? Cienc Cult 69:52-55.
2. Suratman S, Edwards JW, Babina K (2015) Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. Rev Environ Health 30:65-79.
3. Weiss B, Amler S, Amler RW (2004) Pesticides. Pediatrics 113:1030-6.
4. Kosamu I, Kaonga C, Utembe W (2020) A Critical Review of the Status of Pesticide Exposure Management in Malawi. Int J Environ Res Public Health 17:6727.

5. Sturza J, Silver MK, Xu L, Li M, Mai X, Xia Y et al (2016) Prenatal exposure to multiple pesticides is associated with auditory brainstem response at 9months in a cohort study of Chinese infants. Environ Int 92-93:478-485.
6. Naughton SX, Terry AVJ (2018) Neurotoxicity in acute and repeated organophosphate exposure. Toxicology 408:101-112.
7. Dias MB, Prata PHL, Sato AS (2013) Organoclorados, Piretrinas e Piretroides. In: *Toxicologia na Prática Clínica;* Filho ADA, Campolina D, Dias MB. 2nd edition; Belo Horizonte, Folium, 519-26.
8. Santos MAT, Areas MA, Reyes FGR (2007). Piretroides - Uma Visão Geral. Alim. Nutr., Araraquarav 18:339-349.
9. Wolansky MJ, Tornero-Velez R (2013) Critical Consideration of the Multiplicity of Experimental and Organismic Determinants of Pyrethroid Neurotoxicity: A Proof of Concept, Journal of Toxicology and Environmental Health, Part B 16:453-490.
10. Khasigian P (2014) Piretrinas e Piretroides. In: Manual de toxicologia Clínica; Olson KR. 6th edition; Porto Alegre: AMGH.
11. Reis A, Cunha EO, Valle MT, Machado MS, Dallegrave E (2020) Effects of subchronic inhalation exposure to an organophosphorus insecticide compound containing dichlorvos on wistar rats' otoacoustic emissions. Braz J Otorhinolaryngol 88:28-35.
12. Cunha EO, Reis A, Macedo MB, Machado MS, Dallegrave E (2019) Ototoxicity of cypermethrin in Wistar rats. Braz J Otorhinolaryngol 86:587-592.

13. Arora S, Balotra S, Pandey G, Kumar A (2017) Binary combinations of organophosphorus and synthetic pyrethroids are more potent acetylcholinesterase inhibitors than organophosphorus and carbamate mixtures: An in vitro assessment. Toxicol Lett 268:8-16.
14. LaDou J (2016) Saúde ocupacional e ambiental internacional. In: CURRENT/ Medicina Ocupacional e Ambiental. LaDou J, Harrison R. 5th edition. Grupo A.
15. Hernández AF, Gil F, Lacasaña M (2017) Toxicological interactions of pesticide mixtures: an update. Arch Toxicol 91:3211-23.
16. Murakami Y, Pinto NF, Albuquerque GSC, Perna PO, Lacerda A (2017) Intoxicação crônica por agrotóxicos em fumicultores. Saúde em Debate 41:563-576.
17. De Freitas MR, Figueiredo AA, Brito GAC, Leitao RFC, Carvalho Junior JV, Gomes Junior RM et al (2009) The role of apoptosis in cisplatin-induced ototoxicity in rats. Braz J Otorhinolaryngol 75:745-52.
18. OECD (2009) Test No. 413: subchronic inhalation toxicity: 90-day study, OECD guidelines for the testing of chemicals, Section 4. OECD Publishing, Paris.
19. Lorito G, Hatzopoulos S, Laurell G, Campbell KC, Petruccelli J, Giordano P et al (2011) Dose-dependent protection on cisplatin-induced ototoxicity - an electrophysiological study on the effect of three antioxidants in the Sprague-Dawley rat animal model. Med Sci Monit. 17:BR179-86.
20. Probst R, Lonsbury-Martin BL, Martin GK (1991) A review of otoacoustic emissions. The Journal of the Acoustical Society of America 89:2027–2067.

21. Kemp DT, Ryan S, Bray P (1990) A guide to the effective use of otoacoustic emissions. Ear Hear 11:93-105.
22. Church MW, Hotra JW, Holmes PA, Anumba JI, Jackson DA, Adams BR (2012) Auditory brainstem response (ABR) abnormalities across the life span of rats prenatally exposed to alcohol. Alcohol Clin Exp Res 36:83-96.
23. Church MW, Williams HL, Holloway JA (1984a) Postnatal development of the brainstem auditory evoked potential and far-field cochlear microphonic in non-sedated rat pups. Developmental Brain Research 14:23–31.
24. Church MW, Williams HL, Holloway JA (1984b) Brain-stem auditory evoked potentials in the rat: Effects of gender, stimulus characteristics and ethanol sedation. Electroencephalography and Clinical Neurophysiology/Evoked Potentials Section 59:328–339.
25. Church MW, Jen KL, Anumba JI, Jackson DA, Adams BR, Hotra JW (2010) Excess omega-3 fatty acid consumption by mothers during pregnancy and lactation caused shorter life span and abnormal ABRs in old adult offspring. Neurotoxicol Teratol 32:171–81.
26. Church MW, Blakley BW, Burgio DL, Gupta AK (2004) WR-2721 (Amifostine) ameliorates cisplatin-induced hearing loss but causes neurotoxicity in hamsters: dose-dependent effects. J Assoc Res Otolaryngol. 2004;5:227-37.
27. Reis A, Dalmolin SP, Dallegrave E (2017) Animal models for hearing evaluations: a literature review. Rev CEFAC 19:417-428

28. Escabi CD, Frye MD, Trevino M, Lobarinas E (2019) The rat animal model for noise-induced hearing loss. J Acoust Soc Am 146:3692.
29. Gatto MP, Fioretti M, Fabrizi G, Gherardi M, Strafella E, Santarelli L (2014) Effects of potential neurotoxic pesticides on hearing loss: A review. NeuroToxicology 42:24-32.
30. Cao Z, Shafer TJ, Murray TF (2011) Mechanisms of pyrethroid insecticide-induced stimulation of calcium influx in neocortical neurons. JPET 336:197–205.
31. Slotkin TA (2004) Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol 198:132-51.
32. Colborn T (2006) Review A case for revisiting the safety of pesticides: a closer look at neurodevelopment. Environ Health Perspect 114:10-7.
33. Morata TC, Little MB (2002) Suggested guidelines for studying the combined effects of occupational exposure to noise and chemicals on hearing. Noise Health 4:73-87
34. Montanha FP, Pimpão CT (2012) Toxicological Effects of Pyrethroids (Cypermethrin and Deltamethrin) In Fish - Review. Revista Científica Eletrônica De Medicina Veterinária – ISSN: 1679-7353. Ano IX (18).
35. Silver MK, Shao J, Ji C, Zhu B, Xu L, Li M et al (2018) Prenatal organophosphate insecticide exposure and infant sensory function. Int J Hyg Environ Health 221:469-478.
36. Silver MK, Shao J, Li M, Ji C, Chen M, Xia Y et al (2019) Prenatal exposure to the herbicide 2,4-D is associated with deficits in auditory processing during infancy. Environ Res 172:486-494.

37. Körbes D (2009) Toxicidade de agrotóxico organofosforado no sistema auditivo periférico de cobaias: estudo anatômico e funcional. [dissertação] Santa Maria: Universidade Federal de Santa Maria.
38. Taveira BLS, Albuquerque GSC (2018). Analysis of notifications of acute intoxication, by agrochemicals, in 38 municipalities of the state of Paraná. Saúde debate 42:spe4.
39. Brasil. Secretaria de Segurança e Saúde no Trabalho (1998). /NR7. Portaria n° 19. Programa de Controle Médico de Saúde Ocupacional. Diretrizes e parâmetros mínimos para avaliação e acompanhamento da audição em trabalhadores expostos a níveis de pressão sonora elevado.

Table 1. Distortion Product Otoacoustic Emissions (DPOAEs) measurements among experimental groups (n=5 rats/group) and effect size based on pre- evaluation (Cohen's D test).

Frequency	**Group**	**Ear**	**Pre-exposure Amplitude (dB)**	**Post-exposure Amplitude (dB)**	**Cohen's D test**	***p-value**
4kHz	Negative control (water) (n=5)	right	23.0 (13.5 - 28.0)	19.5 (17.0 - 20.0)	0.01	0.785
		left	22.0 (20.0 - 26.0)	20.0 (16.0 - 22.0)	-0.02	0.713
	Positive control (cisplatin) (n=5)	right	14.0 (13.0 – 17.0)	7.0 (2.0 – 12.0)	-1.89	**0.043**
		left	18.0 (16.0 – 23.0)	25.0 (23.0 – 29.0)	0.83	0.345

OP+ PYR (n=5)	right	27.0 (24.0 – 29.0)	16.0 (14.0 – 19.0)	-2.62	0.068
	left	29.0 (17.0 – 30.0)	19.0 (14.0 – 20.0)	-0.79	0.345
OP (n=5)	right	20.0 (18.0 – 24.0)	21.0 (12.0 – 22.0)	-1.08	0.080
	left	20.0 (18.0 – 22.0)	19.0 (16.0 – 20.0)	-0.19	0.893
PYR (n=5)	right	16.0 (13.0 – 20.0)	14.0 (11.0 – 18.0)	-0.46	0.588
	left	18.0 (10.0 – 20.0)	10.0 (9.0 – 15.0)	-0.51	0.102
****p-value**	right	0.099	0.200		

		left	0.690	0.137		
6kHz	Negative control (water) (n=5)	right	37.0 (14.0 - 38.0)	30.0 (30.0 - 37.0)	0.01	0.715
		left	39.0 (30.0 - 40.0)	30.0 (29.0 - 33.0)	-0.84	0.136
	Positive control (cisplatin) (n=5)	right	39.0 (38.0 – 39.0)	23.0 (23.0 – 31.0)	-0.69	0.080
		left	25.0 (20.0 – 25.0)	35.0 (31.0 – 38.0)	1.10	0.080
	OP+ PYR (n=5)	right	39.0 (38.0 – 41.0)	33.0 (33.0 – 35.0)	-1.70	0.068
		left	37.0 (34.0 – 42.0)	35.0 (25.0 – 37.0)	-1.36	0.225

	OP (n=5)	right	35.0 (35.0 – 37.0)	37.0 (30.0 – 38.0)	-0.23	0.686
		left	26.0 (24.0 – 27.0)	37.0 (30.0 – 38.0)	0.97	0.416
	PYR (n=5)	right	36.0 (32.0 – 37.0)	37.0 (33.0 – 37.0)	-0.21	0.892
		left	36.0 (36.0 – 40.0)	30.0 (29.0 – 30.0)	-2.33	**0.043**
	****p-value**	right	0.163	0.339		
		left	0.060	0.405		

8kHz	Negative control (water) (n=5)	right	37.0 (32.0 - 44.0)	42.0 (28.0 - 44.0)	-0,10	0.892
		left	45.0 (44.0 - 46.0)	32.0 (32.0 - 41.0)	-1,61	**0.042**
	Positive control (cisplatin) (n=5)	right	41.0 (38.0 – 42.0)	30.0 (28.0 – 37.0)	-1,07	0.225
		left	28.0 (26.0 – 37.0)	39.0 (32.0 – 42.0)	0,18	0.225
	OP+ PYR (n=5)	right	36.0 (34.0 – 39.0)	38.0 (34.0 – 41.0)	0,19	0.279
		left	32.0 (31.0 – 43.0)	36.0 (27.0 – 43.0)	-0,23	0.273

	OP (n=5)	right	31.0 (28.0 – 44.0)	44.0 (44.0 – 44.0)	0,56	0.416
		left	37.0 (23.0 – 41.0)	43.0 (38.0 – 46.0)	0,18	0.686
	PYR (n=5)	right	35.0 (35.0 – 41.0)	35.0 (28.0 – 47.0)	-0,62	0.893
		left	48.0 (46.0 – 49.0)	31.0 (28.0 – 40.0)	-6,42	**0.043**
	****p-value**	right	0.943	0.474		
		left	**0.012**	0.545		

10kHz	Negative control (water) (n=5)	right	38.0 (38.0 - 44.0)	38.0 (38.0 - 42.0)	0,15	0.715
		left	46.0 (44.0 - 49.0)	41.0 (39.0 - 46.0)	-0,51	0.357
	Positive control (cisplatin) (n=5)	right	41.0 (40.0 – 42.0)	34.0 (27.0 – 35.0)	-2,44	**0.043**
		left	23.0 (22.0 – 24.0)	45.0 (36.0 – 46.0)	1,32	0.080
	OP+ PYR (n=5)	right	38.0 (37.0 – 49.0)	36.0 (27.0 – 43.0)	-0,10	0.498
		left	42.0 (38.0 – 43.0)	35.0 (31.0 – 43.0)	-0,32	0.465

	OP (n=5)	right	36.0 (36.0 – 44.0)	41.0 (32.0 – 42.0)	-0,09	0.893
		left	37.0 (30.0 – 39.0)	34.0 (0.0 – 49.0)	-0,52	0.500
	PYR (n=5)	right	38.0 (36.0 – 41.0)	41.0 (29.0 – 45.0)	-1,18	0.893
		left	43.0 (43.0 – 46.0)	31.0 (0.0 – 43.0)	-10,67	0.078
	****p-value**	right	0.763	0.527		
		left	**0.046**	0.519		

12kHz	Negative control (water) (n=5)	right	41.0 (35.5 – 48.5)	38.5 (35.5 – 45.0)	-0,02	0.500
		left	50.0 (42.0 – 51.5)	37.5 (34.5 – 40.5)	-1,29	0.144
	Positive control (cisplatin) (n=5)	right	41.0 (35.0 – 44.0)	24.0 (19.0 – 28.0)	-2,76	**0.042**
		left	21.0 (13.0 – 29.0)	45.0 (36.0 – 46.0)	1,55	0.080
	OP+ PYR (n=5)	right	39.0 (35.0 – 42.0)	33.0 (31.0 – 39.0)	-0,60	**0.043**
		left	41.0 (40.0 – 43.0)	38.0 (38.0 – 41.0)	-0,51	0.343

	OP (n=5)	right	39.0 (38.0 – 44.0)	42.0 (28.0 – 44.0)	-0,78	0.715
		left	36.0 (14.0 – 43.0)	43.0 (36.0 – 44.0)	0,47	0.345
	PYR (n=5)	right	38.0 (34.0 – 40.0)	42.0 (41.0 – 44.0)	-0,11	0.893
		left	43.0 (42.0 – 44.0)	34.0 (0.0 – 37.0)	-6,13	0.068
	****p-value**	right	0.986	0.293		
		left	**0.041**	0.289		

Data expressed as median and interquartile range (IQR). Legend: OP - organophosphate (commercial insecticide dichlorvos). PYR - pyrethroid (commercial insecticide cypermethrin) . p - statistical significance index. *Wilcoxon Signed Rank test for related samples. **Kruskal-Wallis test for independent samples with Dunn *post hoc* test. Significance fixed at 5% for all analysis.

Table 2. Brainstem Evoked Response Audiometry (BERA) latency measurements among experimental groups (n=5 rats/group) and effect size based on pre-evaluation (Cohen's D test).

Group	Ear	Pre-exposure Latency (ms)	Post-exposure Latency (ms)	Cohen's D test	*p-value
Negative control (water) (n=5)	right	0.40 (0.38 - 0.45)	0.55 (0.35 - 0.60)	0.71	0.500
	left	0.43 (0.38 - 0.50)	0.43 (0.38 - 0.58)	0.34	1.000
Positive control (cisplatin) (n=5)	right	0.65 (0.53 - 0.85)	1.10 (0.93 - 1.15)	1.85	**0.042**
	left	0.53 (0.45 - 0.95)	1.20 (0.75 - 1.20)	1.45	**0.043**
OP+ PYR (n=5)	right	0.53 (0.53 - 0.53)	0.48 (0.38 - 0.80)	1.69	0.465
	left	0.48 (0.48 - 0.53)	0.43 (0.38 - 0.48)	-1.23	0.141

OP (n=5)	right	0.55 (0.55 - 0.60)	0.80 (0.78 - 0.93)	3.19	**0.043**
	left	0.58 (0.55 - 0.58)	0.83 (0.63 - 0.83)	3.08	0.138
PYR (n=5)	right	0.58 (0.55 - 0.68)	0.58 (0.53 - 0.65)	-0.34	0.588
	left	0.60 (0.55 - 0.63)	0.63 (0.55 - 0.65)	0.10	1.000
****p-value**	right	0.064	**0.007**		
	left	0.110	**0.003**		

Data expressed as median and interquartile range (IQR). Legend: OP - organophosphate (commercial insecticide dichlorvos). PYR - pyrethroid (commercial insecticide cypermethrin) . p - statistical significance index. *Wilcoxon Signed Rank test for related samples. **Kruskal-Wallis test for independent samples with Dunn *post hoc* test. Significance fixed at 5% for all analysis.

Table 3. Distortion Product Otoacoustic Emissions (DPOAEs) amplitude measurements among experimental groups (n=5 rats/group).

A. Right ear

Frequency	Negative control (water, n=5)	Positive control (cisplatin, n=5)	OP + PYR (n=5)	OP (n=5)	PYR (n=5)	*GEE		
						Group	Time	Interaction
4 kHz								
Pre[A]	18.40±3.92	15.80±1.78	26.40±1.59	21.40±2.07	17.60±2.44	**0.018**	**0.005**	0.102
Post[B]	18.31±1.07	9.21±2.62 [a]	16.00±2.51 [b]	19.65±2.28 [b]	14.80±2.11			
6 kHz								
Pre	27.60±6.82	36.80±1.99	38.80±1.51	33.20±2.78	31±40.11	0.204	0.500	0.085
Post	31.60±4.82	27.03±2.01	32.40±1.99	31.60±4.42	36.14±0.91			
8 kHz								
Pre	36.40±5.55	39.00±3.52	36.80±2.52	35.60±3.88	35.80±2.30	0.725	0.588	0.368
Post	36.80±4.25	29.60±3.69	38.00±2.10	41.00±3.64	32.20±7.10			
10 Hz								
Pre	36.80±4.44	42.00±1.67	37.40±6.21	36.80±3.53	37.60±1.76	0.955	0.237	0.164
Post	39.80±1.45	31.80±1.99	35.80±4.21	36.00±3.59	32.40±7.52			
12 kHz								
Pre	35.80±6.22	39.60±2.38	37.60±2.79	38.20±3.19	34.40±4.88	0.246	0.355	**0.025**
Post	38.40±2.92	23.20±4.57[a]	33.40±3.63	40.48±3.58	42.72±0.95[b]			

B. Left ear

Frequency	Negative control (water, n=5)	Positive control (cisplatin, n=5)	OP + PYR (n=5)	OP (n=5)	PYR (n=5)	*GEE		
						Group	Time	Interaction
4 kHz								
Pre	19.40±3.48	17.00±3.45	24.20±3.66	20.6±1.69	16.80±3.74	0.834	0.747	0.440
Post	19.40±1.78	24.20±2.30	17.00±2.21	19.8±2.22	14.97±3.36			
6 kHz								
Pre	35.00±2.62	25.60±3.71	36.60±2.36	26.80±2.79	37.00±1.96[A]	0.357	0.952	**0.018**
Post	30.20±1.68	35.80±2.18	28.60±5.05	33.60±3.56	31.92±2.08[B]			
8 kHz								
Pre	44.00±2.48[A]	32.40±3.35[a]	36.40±3.45	33.00±3.91[a]	48.20±1.25[Ab]	0.197	0.556	**≤0.0001**
Post	35.40±2.05[B]	37.40±2.03	34.40±3.66	44.67±2.47	35.63±2.95[B]			
10 kHz								
Pre	44.40±3.23	27.00±4.29[a]	39.20±3.22	32.80±4.92	44.40±0.78[b]	0.109	0.128	**0.018**
Post	41.40±1.89	41.20±3.17	36.60±3.08	44.69±4.35	39.26±3.43			
12 kHz								
Pre	46.40±2.77[Aa]	22.40±4.50[b]	41.00±1.90[a]	31.20±7.44[a]	42.40±1.34[Aa]	0.088	0.226	**0.002**
Post	37.45±1.66[B]	39.80±4.23	38.60±1.78	40.00±2.51	36.60±1.01[B]			

Data expressed as mean ± standard error of mean (SEM). A (right ear) and B (left ear) of 4, 6, 8, 10 and 12 kHz. Legend: n - absolute frequency. kHz – kilohertz. OP - organophosphate (commercial insecticide dichlorvos). PYR - pyrethroid (commercial insecticide cypermethrin). [ab] Different lowercase letters indicate difference among the studied groups in the same time. [AB]

Different uppercase letters show the evolution of a certain group over time. *Gamma with log link Generalized Estimate Equation test (Bonferroni *post hoc*). Significance set at 5% for all analyses.

Figure 1.

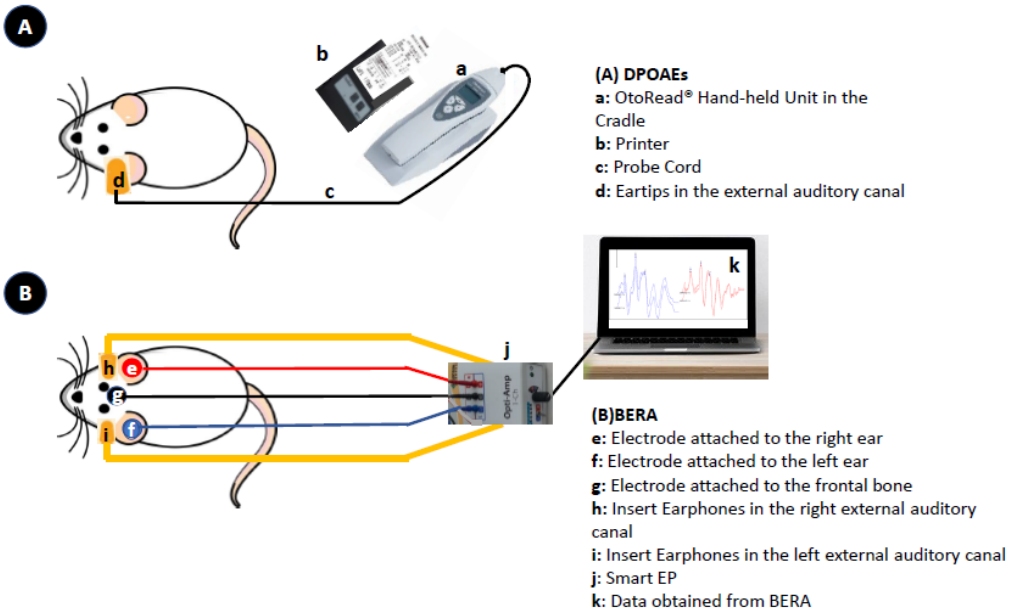

Figure 1: Schematic representation of the auditory assessment by Distortion Product Otoacoustic Emissions - DPOAEs (A) and Brainstem Evoked Response Audiometry – BERA (B)

Figure 2.

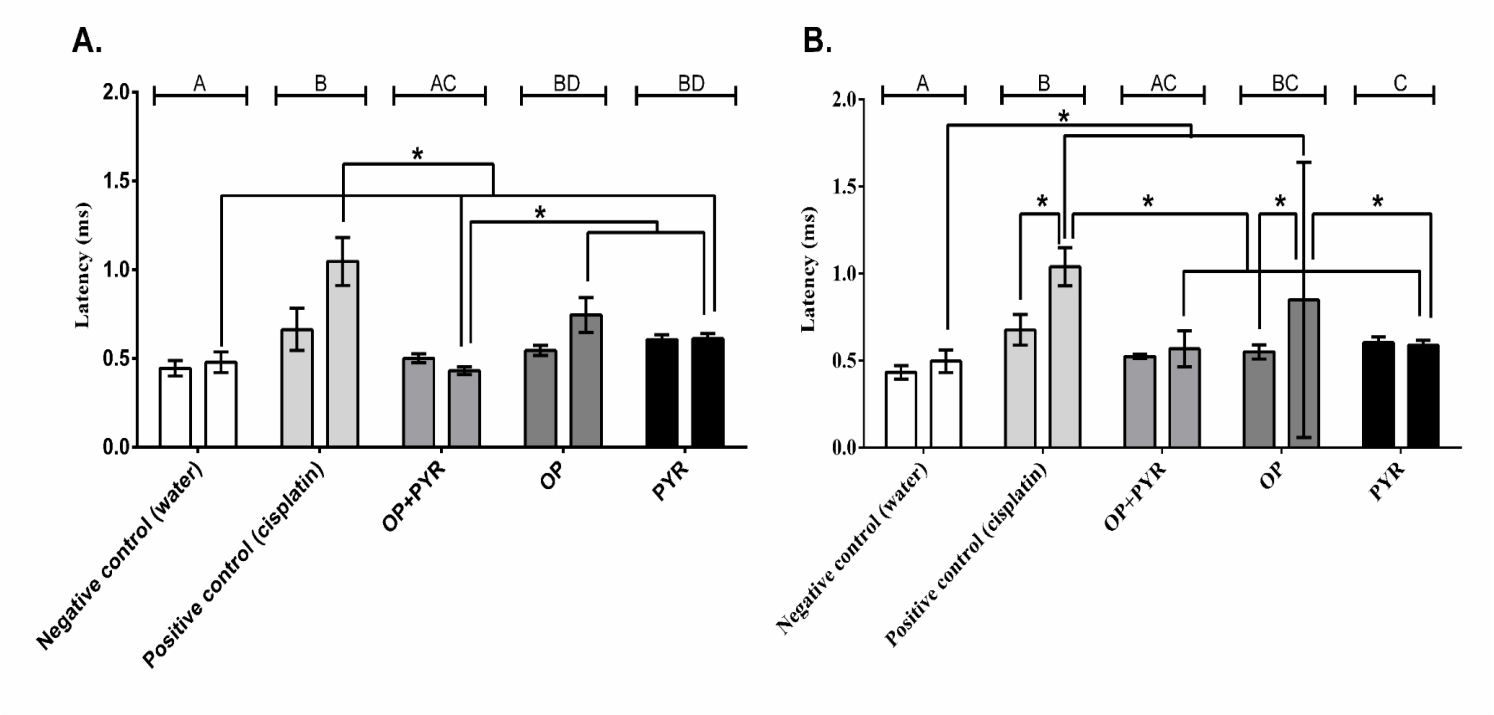
A.
A
B
AC
BD
BD
2.0
1.5
1.0
0.5
0.0
Latency (ms)
*
*
Negative control (water)
Positive control (cisplatin)
OP+PYR
OP
PYR
B.
A
B
AC
BC
C
2.0
1.5
1.0
0.5
0.0
Latency (ms)
*
*
*
*
*
Negative control (water)
Positive control (cisplatin)
OP+PYR
OP
PYR

FIGURE CAPTIONS

Figure 1. Schematic representation of the auditory assessment by Distortion Product Otoacoustic Emissions - DPOAEs (A) and Brainstem Evoked Response Audiometry – BERA (B).

Figure 2. Brainstem Evoked Response Audiometry (BERA) latency measurements among experimental groups (n=5 rats/group). Data expressed as mean ± standard error of mean (SEM); each bar represents one evaluation (pre or post exposure). A (left ear) and B (right ear). BERA latency (in milliseconds, ms). Legend: OP - organophosphate (commercial insecticide dichlorvos). PYR - pyrethroid (commercial insecticide cypermethrin). OP + PYR – organophosphate plus pyrethroid. Gamma with log link Generalized Estimating Equations (GEE) model with Bonferroni correction. ^{AB} Different letters indicate group effects. * Indicate group and time interaction. Significance set at 5% for all analysis.

8. CONCLUSÕES

No primeiro projeto, houve mudança nas amplitudes das EOAPD dos animais expostos a associação de organofosforado e piretróide. Não houve diferença significativa no desenvolvimento ponderal dos animais expostos aos agrotóxicos. Durante o período de exposição aos pesticidas, os animais do grupo experimental não mostraram alteração nos sinais clínicos (exceto nos 30 minutos iniciais dos dois primeiros dias de exposição). Não houve diferença na colinesterase plasmática do grupo associação. Não houve diferença na massa relativa dos órgãos dos animais expostos aos agrotóxicos.

No segundo projeto, houve mudança nas amplitudes das EOAPD dos animais expostos ao organofosforado diclorvós, piretróide cipermetrina e sua associação. Houve aumento da latência da onda P_{II} no grupo exposto ao organofosforado. Não houve diferença significativa no desenvolvimento ponderal dos animais expostos aos agrotóxicos. Durante o período de exposição aos pesticidas, os animais do grupo OF, PIR e associação não mostraram alteração nos sinais clínicos (exceto nos 30 minutos iniciais dos dois primeiros dias de exposição). Não houve diferença na massa relativa dos órgãos dos animais expostos aos agrotóxicos. O segundo projeto demonstrou que o piretróide agiu predominantemente na função coclear por meio da redução da amplitude das emissões otoacústicas produto de distorção. O organofosforado, por sua vez, afetou principalmente as fibras do nervo auditivo ao aumentar a latência da onda P_{II} no exame de potencial evocado auditivo de tronco encefálico. Quando ambos os pesticidas foram combinados, os efeitos da associação tornaram-se menores do que os de cada um dos ingredientes isolados, independentemente da região anatômica avaliada. Embora o grupo

associação não tenha apresentado prejuízo audiológico no momento da avaliação, foram observados efeitos individuais dos agrotóxicos estudados. Esse cenário evidencia a necessidade da aplicação de testes auditivos complementares na avaliação de indivíduos expostos a agrotóxicos, além dos testes normalmente utilizados nas avaliações audiológicas de rotina.

Houve ototoxicidade secundária à exposição aos agrotóxicos diclorvós, cipermetrina e sua associação, sem toxicidade sistêmica relevante.

9. CONSIDERAÇÕES FINAIS

Ambos os projetos descritos nesta tese foram desenvolvidos no Laboratório de Pesquisa em Toxicologia (Lapetox) da Universidade Federal de Ciências da Saúde de Porto Alegre. A colaboração entre as áreas de toxicologia e fonoaudiologia teve início no ano de 2015, com o aceite de orientação da Profa. Dra. Eliane Dallegrave a um projeto de mestrado da fonoaudióloga Aléxia dos Reis. Desde então uma linha de pesquisa em ototoxicidade foi criada e vários estudos acerca do tema estão publicados ou em fase de análise para publicação. O objetivo do Lapetox ao trabalhar com este tema é aumentar a consciência da comunidade científica, dos trabalhadores do meio agrícola, da população em geral e dos legisladores acerca dos danos que os agrotóxicos podem causar no sistema auditivo.

O presente trabalho possui algumas limitações potenciais. Embora o dano auditivo causado por agentes ototóxicos seja similar ao apresentado na presente pesquisa, existe escassez de estudos prévios que elucidem o mecanismo específico da toxicidade por meio de correlação dos resultados de avaliações objetivas da audição com dados morfológicos em estruturas como células ciliadas e nervo auditivo. Nesse sentido, novas pesquisas que associem tais dados proporcionariam um entendimento melhor da fisiopatologia do dano, bem como a descoberta de substâncias que tenham atividade otoprotetora. Em ambos os estudos foi possível observar ototoxicidade secundária à exposição aos agrotóxicos diclorvós e cipermetrina, sem toxicidade sistêmica relevante.

Os resultados apresentados nesta tese demonstram que existe um grande potencial para ototoxicidade após a exposição a agrotóxicos. A legislação brasileira, no entanto, no momento não considera tal dano, uma vez

que os trabalhadores expostos a essas substâncias não realizam controle audiométrico de acordo com a Norma Reguladora 7. Um dos testes que a legislação prevê para os trabalhadores que têm contato com pesticidas é o de atividade da colinesterase plasmática, porém os dados da presente tese demonstram que – na concentração pesquisada – não houve alteração desse biomarcador, mesmo com a ototoxicidade já presente. Nesse sentido, há perspectiva de que mudanças da legislação para melhor proteção da saúde dos trabalhadores se fazem necessárias.

10. APÊNDICE

Artigo desenvolvido pelo grupo de pesquisa Laboratório de Pesquisa em Toxicologia (Lapetox) da Universidade Federal de Ciências da Saúde de Porto Alegre (Journal of Voice).

ARTICLE IN PRESS

Morphometric Evaluation of the Recurrent Laryngeal Nerve of Wistar Rats Exposed to Pesticides

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ABSTRACT: The literature has been shown that exposition by inhalation to chemical compounds can cause vocal disorders and dysphagia in humans, in addition to other symptoms that are manifested according to the type, concentration and duration of exposure to the substance. Cypermethrin and dichlorvos are pesticides widely used in agriculture, public health, veterinary, and home environments. Despite the scientific evidence that cypermethrin and dichlorvos can cause neurodegenerative damage and motor alterations, there are no studies evaluating the toxic effects of these pesticides on the morphology of structures responsible for vocal mobility, especially to the Recurrent Laryngeal Nerve (RLN). Considering the association between vocal disorders in humans and variations in RLN and morphometry, the aim of this study was to evaluate the possible alterations in the microstructure of RLN secondary to subchronic exposure to cypermethrin (pyrethroid) and dichlorvos (organophosphate) in Wistar rats. The experimental protocol (approved by CEUA-UFCSPA: 321/15 and 323/15) consisted of 15 male Wistar rats, allocated in 3 groups: Control (n = 5, exposed to water), Cypermethrin (n = 5, exposed to cypermethrin - 1/10 of the inhalation median lethal concentration [LC_{50}] - 0.25 mg/L) and dichlorvos (n = 5, exposed to dichlorvos - 1/10 of the LC_{50} - 1.5 mg/L). Inhalation exposure was performed for 4 hours, 5 times per week, for 6 weeks. The nerves were collected, histologically processed and analyzed using morphometric parameters measured using ZEN 2.6 (Zeiss – Germany). The cypermethrin and dichlorvos groups showed significant changes ($P < 0.001$, ANOVA) in the g-ratio and in the thickness of the myelin sheath of the RLN when compared to the control animals, however, none of the other parameters evaluated showed statistically significant differences. These findings indicate that repeated inhalation exposure to commercial products of cypermethrin and dichlorvos is able to modify the structure of the RLN and possibly generating vocal changes and / or dysphagia.

Key Words: Dichlorvos—Cypermethrin—Recurrent Laryngeal Nerve—Inhalation exposure—G-ratio—Rats.

INTRODUCTION

Although the literature has indicated that certain chemical compounds are harmful to airways with potential neurotoxic effects,[1,2] there is still a lack of information about possible vocal changes resulting from inhalation exposure to these substances.[3,4] Moreover, the few studies have described in this area are case reports that do not consider the possibility of voice disorders arise from neurotoxic effects related to nerves vocal production, thus keeping their discussion focused on the respiratory tract impacts.[5,6]

In general, individuals exposed by inhalation to chemical compounds may present shortness of breath, cough, tension in the cervical region, hoarseness, rhinitis, dysphagia for liquid and solids, dyspnea, throat irritation, pharyngitis, laryngitis and vocal fold dysfunction. Moreover, it is described that symptoms are manifested according to the type, concentration, and time of exposure to the substance.[1,5–9]

Among the most widely used pesticides, organophosphate insecticides, and pyrethroids are generally represented in the literature involving poisoning cases.[3,10,11] Dichlorvos is an organophosphate pesticide, used to fight insects in household environment, commercial and industrial buildings, health facilities, schools, as well as public health in order to control vectors.[12,13] The neurotoxic effects of organophosphate compounds occur by inhibiting the acetylcholinesterase enzyme that generates the accumulation of neurotransmitter acetylcholinesterase in the synaptic cleft.[14,15] Consequently, the intoxication can cause 3 distinct neurological syndromes: acute cholinergic crisis, intermediate syndrome, and organophosphate-induced retarded polyneuropathy.[2,16] There is evidence in the literature about the occurrence of vocal fold paralysis due to organophosphate poisoning in humans.[17–20]

The cypermethrin is a pyrethroid type II pesticide that is mainly used in agriculture, veterinary medicine, public health, and in home environments. It is worldwide used and considered of high efficiency and low toxicity in mammals.[21–23] Pyrethroids are able to prolong the opening of sodium ion channels causing hyperexcitability, thus being neurotoxic agents acting in axon transmission.[24] Due to its low environmental risk factor, it was applied as an

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alternative to the substances with the highest potential toxicity.[25,26] Although these pesticides show less risk compared to others, there is still much evidence to problematize their indiscriminate use. Studies carried out on animal model concluded that pesticides exposure induces hematological and histopathological changes in the brain,[24,27–29] and on the motor control of Wistar rats.[29–31]

The relationship between pesticides inhalation and neurotoxicity is still slightly explored in studies, and most of the animal models research uses other routes of administration (oral, subcutaneous, intramuscular, intraperitoneal, and percutaneous) not mimicking the occupational inhalation exposure in which the individuals who do not always adhere the use of protective equipments.[2,32–34] Furthermore, the evidences so far is not dedicated to the assessments of neurotoxic effects of inhaled exposure to pesticides on the peripheral innervation responsible for vocal production. Nevertheless, the literature shows peripheral neurotoxicity by exposure to pesticides.[2,32,35,36]

In this context, considering the lack of research involving neurotoxic aspects in the laryngeal nerves, we decided in an unprecedented way to evaluate the histomorphometry changes of the Recurrent Laryngeal Nerve (RLN) secondary to subchronic inhalation exposure to pesticides dichlorvos, and cypermethrin in Wistar rats.

MATERIAL AND METHODS

This experimental study was based on the inhalation subchronic toxicity assessment protocol, number 413, according to the Organization for Economic Co-operation, and Development (OECD) - Guideline for chemical testing.[22] All animal procedures were approved by the Animal Use Ethics Committee of University of Health Sciences of Porto Alegre (UFCSPA) under n° 321/15, 323/15.

Animals

Fifteen 60-day-old male Wistar rats (*Rattus norvegicus*) were used, weighing approximately 300 + 50g and kept under controlled bioterium conditions with 12h light/dark cycle, receiving feed and water *ad libitum*, except at the time of exposure to pesticides.

Exposure

The animals were randomly allocated in 3 groups: Control [n = 5, exposed to water (vehicle for formulation dilution)]; Organophosphate [n = 5, exposed to 1/10 LC_{50} (1.5 mg/L) of Dichlorvos (Organophosphate - commercially formulated -DDVP)]; and Pyrethroid [n = 5, exposed to 1/10 LC_{50} (0.25 mg/L) of Cypermethrin (Pyrethroid - commercially formulated concentrated emulsion)]. All groups were exposed by inhalation, during 4 hours daily, 5 times a week for 6 weeks.

Exposure to Dichlorvos, Cypermethrin and water were performed into a closed system composed of nebulizers (Inalar Compact®) connected to the upper and lateral of the inhalation exposure boxes (56L capacity) and aspirators coupled to their contralateral lower. All flows were regulated and standardized.

Euthanasia

At the end of the experimental period (24h after the last inhalation exposure), euthanasia was performed by previous anesthesia with sodium thiopental (50mg / kg) associated to intraperitoneal lidocaine (10mg / kg). Blood was collected from the cava vein and used to assess plasma butyrylcholinesterase (organophosphate exposure biomarker).

Recurrent Laryngeal Nerve collection

The anterior cervical region was dissected, opening the structures flat to flat, deep towards the Recurrent Laryngeal Nerve. Through a stereomicroscope, it was extracted approximately 4mm fragment from the left Recurrent Laryngeal Nerve. All procedures described here were based on previous studies.[37,38]

Histology

Recurrent Laryngeal Nerve fragments were embedded in fixative solution [0.5% glutaraldehyde (Sigma Chemicals Co., St Louis, MO, USA) and 4% paraformaldehyde (Reagen, Brazil)] in phosphate buffer. After that, the fragments were post-fixed in 1% OsO4 (Sigma Chemicals Co), dehydrated at increasing concentrations of acetone (Dinâmica, Brazil), soaked in acrylic resin (Durcupan, ACM-Fluka, Switzerland) and polymerized at 60°C. Five transverse sections (1 μm) were obtained using an ultramicrotome (Leica, Nussloch, Germany) at 100 μm intervals, on slides, and stained with 1% toluidine blue (Merck, Farmstadt, Germany) in 1% tetraborate sodium (Ecibra, Curitiba, Brazil).

Histomorphometric analysis

Recurrent Laryngeal Nerve analyzes were based on previous studies.[38] The images of the nerves were captured using an Axio Imager.Z2 microscope (Zeiss - Germany) which has a digital camera AxioCam connected to Zen 2.6 software (Zeiss -Germany). Images were analyzed using Zen 2.6 software (Blue edition– Zeiss – Germany).

According to the previous planar morphometry protocol,[37,39] were analyzed:

- The mean density of the myelinated nerve fibers.
- The average intraperineural area.
- Estimative of the total number of myelinated fibers.
- The mean of the area of myelinated fibers.
- The total mean of axon area.
- Diameter of myelinated fibers mean.
- The axonal diameter mean.
- The mean of the thickness of the myelin sheath.
- The mean of the myelinization degree (g-ratio).
- The percentage of the total area occupied by the myelinated fibers.

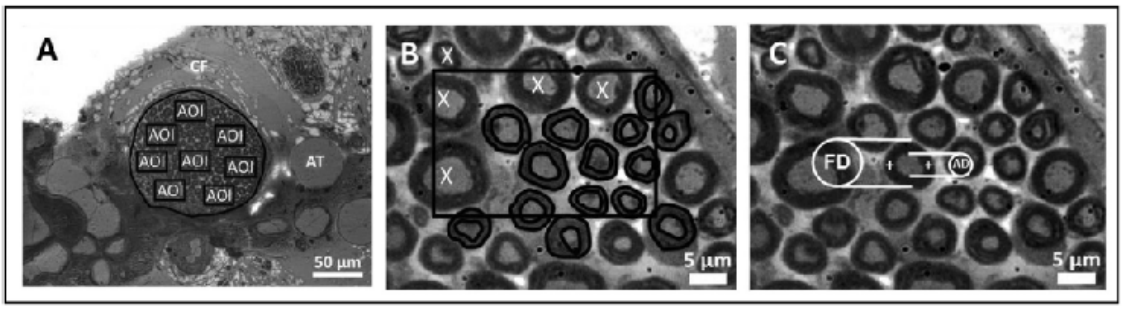

FIGURE 1. Planar morphometry protocol. Image A presents a cross-section of the RLN that demonstrates the allocation of the 8 areas of interest (AOI) and the respective delimitation of the intraperineural area in a final magnification of 400x. In B, the method of measuring the area of the myelinated nerve fibers and the axonal area, demonstrated through the delineation of the nerve fibers at a final magnification of 1,000x, is represented. Furthermore, it can be observed that the fibers containing an "x" were not measured for touching the AOI exclusion bar. In C, the diameter of the myelinated fiber (DF) and the axonal diameter (AD) are represented, followed by the signaling from the myelin sheath of a nerve fiber demonstrated by the "+" signs at a final magnification of 1,000x. AT, adipose tissue, CF, collagen fibrils.

- The percentage of the total area occupied by the endoneurial connective tissue.

The planar morphometry protocol can be better understood through Figure 1, which illustrates the measurements performed. The histomorphometric variables analyzed, except the intraperineural area, were evaluated using 8 Area of Interest (AOI) measuring 600 μm^2 allocated to central, lateral, upper, and lower nerve portions in the scanned image with a final increase of 1,000x, totalizing 24,000 μm^2 of RLN per group.

Fiber density was obtained from the following equation: total number of fibers counted in all AOI / analized area. The intraperineural area were measured from 1 cross section of the nerve with magnification of 400x, from the intraperineural delineation the total fiber in each nerve was estimated by multiplying the fiber density by the intraperineural area of RLN.

Axonal area was measured from fiber design without including the myelin sheath and the area of the myelinated fiber was measured and obtained from the myelin sheath itself. From these data, the respective diameters were estimated from the conversion by the equivalent area of a circle, and its respective diameter value.[40]

The myelin sheath thickness (MST) was estimated by the axon perimeters (AP) and myelinated fiber perimeters (MFP) using the following formula:[41]

$$MST = \frac{(MFP - AP)}{(2\pi)}$$

The g-ratio was estimated for each nerve fiber measured in the AOI from a division between the axonal diameter by the diameter of the myelinated nerve fiber. Afterwards, the average of the g-ratio of several fibers per animal was calculated, and compared by ANOVA between the groups.

The percentage of the total area occupied by myelinated nerve fibers was estimated through the following equation: total area occupied by myelinated fibers / intraperineural area x 100.

The percentage of the total area occupied by endoneurial connective tissue were calculated by the following subtraction: 100% - the percentage of the total area occupied by myelinated nerve fibers.

Statistical analysis

The histomorphometric parameters evaluated from the Recurrent Laryngeal Nerve of the control, cypermethrin and dichlorvos groups were represented as mean and standard error mean and compared independently by the one-way ANOVA test followed by the Bonferroni post hoc, considering a 95% confidence. All statistical analyzes were performed using Microsoft Excel 16.0 (Microsoft - USA) Statistical Software Package (SPSS version 21.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

The cross sections of the left unilateral Recurrent Laryngeal Nerve (RLN) innervation, stained with toluidine blue, is showed in Figure 2. The histomorphometric analysis demonstrated well-defined limits, easily observed under the optical microscope.

Results of morphometric parameters are summarized in Figure 3A-3K. Only 2 parameters were found to be significantly different from controls g-ratio [$P = 0.0001$; 1-way ANOVA, Bonferroni (Figure 3I)] and the Dichlorvos group showed alteration of thickness myelin sheath [$P = 0.009$; 1-way ANOVA, Bonferroni (Figure 3H)].

No statistically significant differences (Figure 3A-3G and 3J-3K) were found for the other parameters [Density of the myelinated nerve fibers ($P = 0.509$); Intraperineural area ($P = 0.095$); Number of myelinated fibers/nerve ($P = 0.352$); Myelinated fibers area ($P = 0.367$); Axon area ($P = 0.338$); Myelinated fibers diameter ($P = 0.367$); Axonal diameter of myelinated nerve fibers ($P = 0.414$); Percentage of the myelinated fibers by occupied area ($P = 0.817$); Percentage of

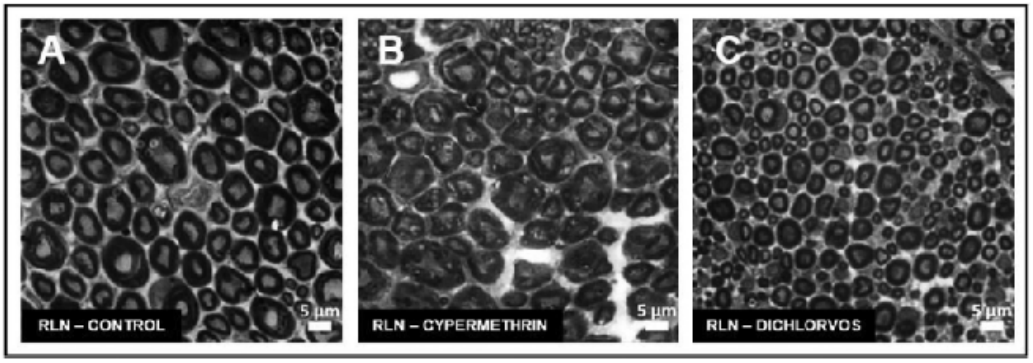

FIGURE 2. Histomorphometric analysis of the cross sections of the left unilateral recurrent laryngeal nerve of rats after water, cypermethrin, or dichlorvos subchronic inhalation exposure. Images A, B, and C show the cross sections of the recurrent laryngeal nerve in a final magnification of 1,000x.

the endoneurial connective tissue by occupied area (P = 0.817)]. Furthermore, plasma butyrylcholinesterase did not present a statistically significant difference when compared to the control group [P = 0.73; 1-way ANOVA (data not shown)].

DISCUSSION

Inhalation is the main route of exposure to chemicals such as pesticides.[42,43] However, most of the evidence found in the literature used intraperitoneal, and oral routes in their experiments involving these substances.[27,28,30,44,45] So, this study is a pioneer in assessing damage to the RLN myelin sheath by inhaled subchronic exposure to cypermethrin and dichlorvos.

The pesticides concentration used in this study was lower than the mentioned in the previous studies - one tenth of the inhaled lethal concentration for Wistar rats - LC50.[25] Nonetheless, it was able to cause an increase in the degree of myelination (g-ratio), and the concentration of dichlorvos was also able to change the thickness of the myelin sheath. It is a concern, since humans are exposed to higher concentrations, and for longer daily exposure periods than that used in this study (4h per day). In this context, it could be exemplified by the use in indoor environments, such as domestic ones, in which cypermethrin can stay for more than 30 days on the surface of furniture, walls, and floors.[46] This study has provided evidence that vocal changes in humans may be related to changes in the morphological structure of RLN due to inhalation exposure to pesticides.

Although the literature does not provide specific data about our results, we will discuss in the next sections about the neurotoxicity mechanisms of pesticides, the damage in the peripheral nervous system, and the vocal pathophysiology implications.

Neurotoxic mechanism of pesticides

Pesticides in general, including organophosphates, and pyrethroids can be neurotoxic.[2,15] Exposure to pesticides such as organophosphate class, exemplified in this study by dichlorvos, can inhibit the action of the enzyme acetylcholinesterase, causing neurotoxicity by the mechanism of postsynaptic superstimulation of cholinergic receptors.[3,14,47,48] Organophosphate poisoning is well established in the literature and can generate 3 distinct neurological syndromes: acute cholinergic crisis, intermediate syndrome and organophosphate-induced delayed polyneuropathy.[6,34] In humans, the less severe cases of acute cholinergic crisis of organophosphate poisoning described in the literature report the beginning of symptoms such as headache, dizziness, nausea, vomiting, pupillary constriction, sweating, tearing, and salivation.[34] In more severe cases, symptoms of muscle weakness and spasms, bronchospasms and changes in heart rate that can progress to seizures and coma are reported in studies.[2,34]

The intermediate syndrome, characterized by the appearance of symptoms of intoxication 1 to 4 days after exposure, usually presents with muscle weakness, which can be fatal if the respiratory muscles are compromised.[34]

Delayed polyneuropathy induced by organophosphates is a syndrome characterized by involvement of the sensory component, muscle cramps, weakness, and even muscle paralysis.[34] The picture generated by polyneuropathy is a consequence of axonal death and can be irreversible due to the inhibition of neural enzyme called neuropathy target esterase.[2,34]

Organophosphate poisoning has additional long-term sequelae, including some studies showing the emergence of changes after ten years of exposure, such as deficits in cognitive and psychomotor function, decreased sensitivity and motor dysfunction.[34,49]

Cypermethrin, the pesticide used in this study, is a type II pyrethroid that can prolong the opening of sodium channels in the nervous system leading to hyperpolarization and hyper-excitation of neurons, thus, neurotoxic agents acting in axon transmission.[50,51] It has also been described in the literature that cypermethrin generates nerve conduction block, reducing the amplitude of the action potential,[52,53] also inducing neurotoxicity by modulating the level of gamma-aminobutyric acid – GABA.[54,55]

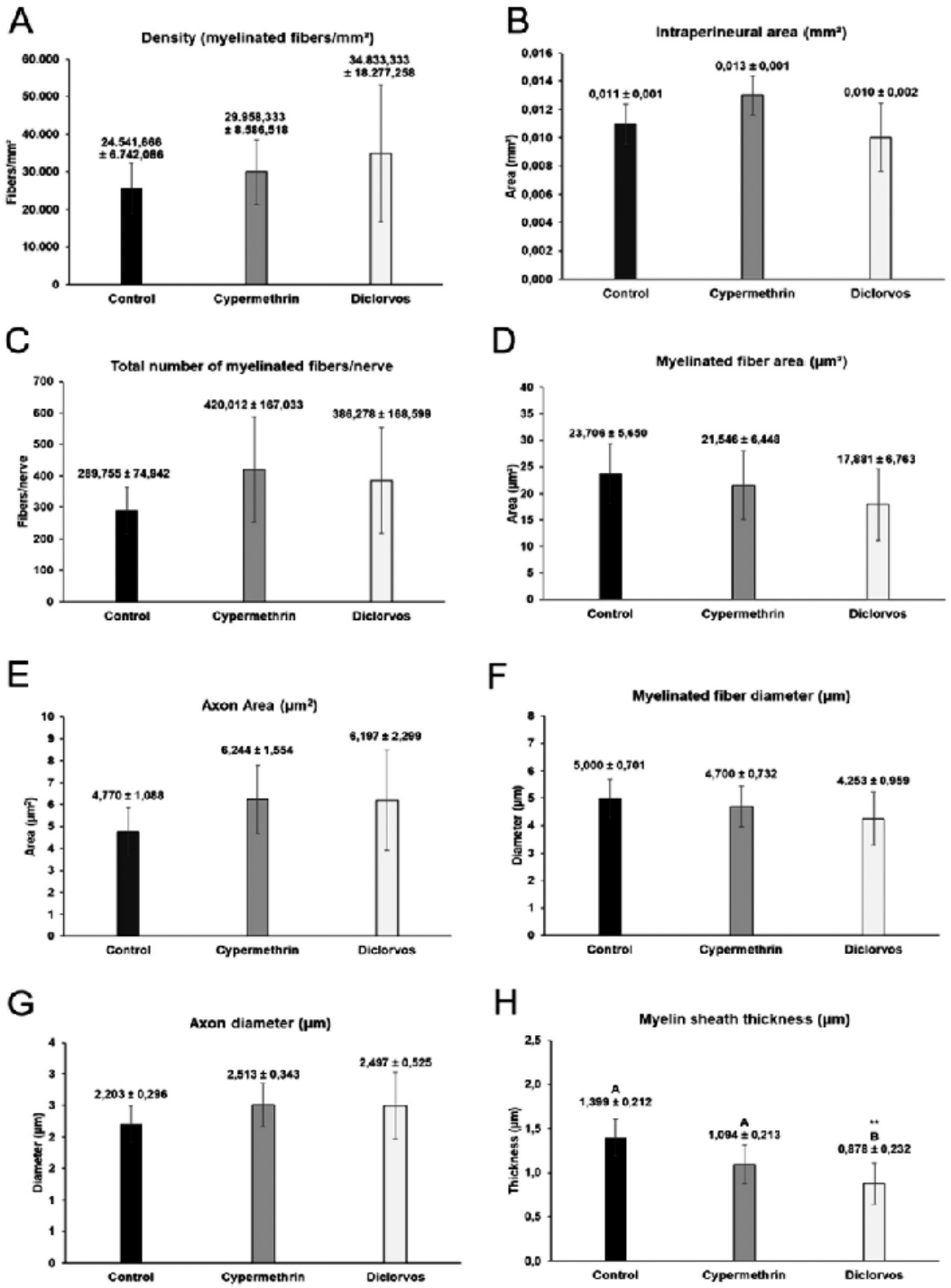

FIGURE 3. Comparison of myelinated fibers density A. intraperineural area B. total number of myelinated fibers C. myelinated fiber area D. axon area E. myelinated fiber diameter F. axon diameter G. myelin sheath thickness H. *g*-ratio I. area of myelinated fibers J. and area of connective tissue K. between the RLN of group control, cypermethrin, and dichlorvos. $^{**}P < 0.05$ and $^{***}P < 0.001$ (ANOVA). Different letters indicate significant differences between groups (Bonferroni post hoc test).

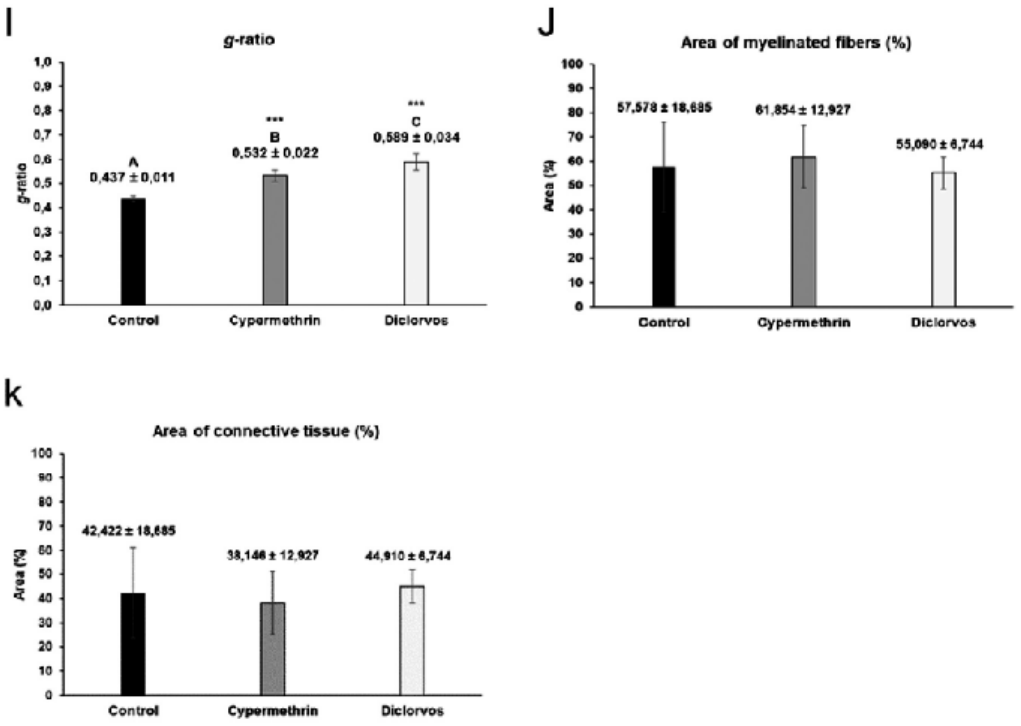

FIGURE 3. Continued

Pyrethroids are considered less toxic to mammals when compared to other classes of pesticides,[21–23] however, studies do not assess their neurotoxicity by chronic exposure isolated in humans.[34,49] In this scenario, it is important to consider that individuals are frequently exposed to many pesticides or mixtures of different pesticides, simultaneously or in series. These exposures problematize the identification of the effects of some agents in humans.[34,49]

In this context, the peripheral neurotoxicity described by the morphometric parameters of this study is extremely important for a better understanding of the neurotoxic effects of these substances. The use of animal models is essential, as it can help to better understand the findings of research with humans exposed to pesticides, since they allow controlling possible biases.[56]

Effects of pesticides on the peripheral nervous system

The literature has not presented enough evidence that assess the effects of inhalation pesticides in the peripheral nervous system.[2,32,34,56] Studies in humans exposed to pesticides show that some organophosphorus compounds are capable of causing damage to peripheral nerve conduction.[34] On the other hand, considering the other pesticide classes, there is still need for further studies.[34]

Research involving animal models seems to corroborate the findings in humans. A study carried out in rats evaluated the electrophysiology and histopathology of the respiratory tract, diaphragm, and phrenic nerve after inhalation of dichlorvos. The data obtained in this research demonstrated that the inhalation of the organophosphate compound caused neuropathy with involvement of the diaphragm and phrenic nerve, in addition to damage to the respiratory tract.[32] Another study also investigated the damage to the nervous system after exposure to pesticides in rats. For this, histopathological damage in the brain, spinal cord and sciatic nerve after subchronic ingestion of an organophosphate, a carbamate and their mixture were evaluated. The result of the study showed central and peripheral neurotoxicity in the evaluated animals.[36]

Although there are no studies as our research that evaluated the damage to laryngeal motor innervation secondary to inhaled exposure to pesticides, few studies involving other models show recurrent laryngeal nerve paralysis after exposure to organophosphate compounds. The studies cite the occurrence of recurrent laryngeal nerve paralysis after ingestion of haloxon, an anthelmintic composed of organophosphate, in foals, pigs, and horses.[20,57,58] Two other studies involving cats that experimentally received an organophosphate compound by intraperitoneal route developed axonal degeneration in the recurrent laryngeal nerve.[59,60]

Implications for pathophysiology of vocal function

Only a few studies describe exposure to pesticides and vocal problems.[3] From this perspective, it is important to consider that pesticide poisoning cannot always be diagnosed, especially among rural workers with limited access to the health system.[36] Furthermore, most studies that report vocal problems describe cases of acute intoxication by ingestion of organophosphate compounds in suicide attempts, in other words, cases with high severity that resulted in vocal fold paralysis.[18,20] It is relevant to highlight that medium cases of vocal alterations caused by pesticides possibly do not reach medical attention, as individuals may minimize these symptoms by not using their voice professionally.

In the researched literature, only one study involving the analysis of medical records reported the symptoms of vocal alterations in workers exposed to pesticides chronically. In this study, hoarseness was the main symptom described and the pesticides involved belonged to the class of organophosphates and pyrethroids.[3] The data obtained in our study corroborated these findings, as both classes of pesticides mentioned are present in our experimental model, which showed damage to the laryngeal motor innervation that could possibly result in vocal changes.

Even though our study only provides evidence on neurotoxicity for vocal motor function, we must consider that the nerves are generally affected, so the laryngeal sensory innervation is also damaged, and may contribute to vocal problems in individuals exposed to pesticides. In this context, our inferences can be corroborated by other studies involving the assessment of sensory nerves after exposure to pesticides with consequent development of neuropathy.[61,62]

It is important to recognize that the present study showed significant increase in the degree of myelination (g-ratio) of the RLN of rats exposed to dichlorvos and cypermethrin, as well as a reduction in the thickness of the myelin sheath of rats exposed to dichlorvos by subchronic inhalation route, which represents alterations that could affect voice. The other measurements did not show a significant difference, possibly due to the low concentration of pesticides used, corroborated by the absence of butyrylcholinesterase reduction in the exposed animals.[48] According to these results, the g-ratio seems to be a more sensitive measurement to assess the change in RLN induced by subchronic inhalation exposure to these pesticides.

The g-ratio is widely applied as a functional and structural index of peripheral axonal myelination.[38] This measure is important when evaluating the recovery process of demyelinating fibers.[63,64] Than, it can be used to assess the relationship between nerve conduction velocity, and peripheral nerve fiber morphology during its regeneration.[65] Likewise, other studies[39,66] used g-ratio as an important parameter for the assessment of the degree of myelinization of peripheral nerve fibers in the field of microsurgery.[67]

The reduction of the thickness of the myelin sheath affects the speed of nerve conduction and can cause changes in nerve impulse transmission, generating differences in the firing frequency of action of the thyroarytenoid muscle fibers, and, consequently, in the rate that the vocal folds open and close - glottic pulse.[68] The literature also describes that the demyelination of the RLN is associated to vocal fold paralysis.[69]

This study has provided evidence that the pesticides cypermethrin and dichlorvos in low concentrations can affect the histomorphometry of the RLN. The results obtained here complement the studies involving the neurotoxicity of pesticides in Wistar rats as it provides information regarding their action on the myelin sheath of a peripheral nerve, essential for vocal mobility, protection of the airways during swallowing, and breathing.

CONCLUSIONS

Subchronic inhalation exposure to cypermethrin at a concentration of 0.25 mg/L and dichlorvos at a concentration of 1.5 mg/L for 4 hours daily, 5 times a week for 6 weeks, increased the degree of myelination of RLN, and also dichlorvos reduced the thickness of the myelin sheath of RLN when compared control group. It is the first study to bring data on subchronic inhalation to cypermethrin and dichlorvos using commercial formulations and their effects on the myelin sheath of a peripheral nerve fundamental for vocal mobility, simulating an occupational exposure using animal model. Moreover, this work has provided subsidies for new research involving pesticides and their association with vocal and swallowing disorders. The data obtained here corroborate the others by pointing out that inhalation exposure to cypermethrin and dichlorvos may be a public health problem.

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REFERENCES

1. Kim S, Seo M, Na M, et al. Investigation on combined inhalation exposure scenarios to biocidal mixtures: biocidal and household chemical products in south korea. *Toxics*. 2021;9:1 12. https://doi.org/10.3390/toxics9020032.
2. Richardson JR, Fitsanakis V, Westerink RHS, et al. Neurotoxicity of pesticides. *Acta Neuropathologica*. 2019;138:343–362. https://doi.org/10.1007/S00401-019-02033-9.
3. Lisbôa CD, Mello MG, da S. Voice and speech signs and symptoms in individuals exposed to chemical agents: an analysis of medical records. *Revista CEFAC*. 2018;20:209–217. https://doi.org/10.1590/1982-0216201820214717.
4. Williams NR. Occupational voice disorders due to workplace exposure to irritants - A review of the literature. *Occup Med*. 2002;52:99–101. https://doi.org/10.1093/occmed/52.2.99.
5. Perkner JJ, Fennelly MKP, Balkissoon R, et al. Irritant-associated vocal cord dysfunction. *J Occup Environ Med*. 1998;40:136–143. https://doi.org/10.1097/00043764-199802000-00009.

6. Allan PF, Abouchahine S, Harvis L, et al. Progressive vocal cord dysfunction subsequent to a chlorine gas exposure. *J Voice*. 2006;20:291–296. https://doi.org/10.1016/j.jvoice.2005.04.003.
7. Sala E, Hytönen M, Tupasela O, et al. Occupational laryngitis with immediate allergic or immediate type specific chemical hypersensitivity. *Clin Otolaryngol Allied Sci*. 1996;21:42–48. https://doi.org/10.1111/j.1365-2273.1996.tb01023.x.
8. Lin N, Rosemberg MA, Li W, et al. Occupational exposure and health risks of volatile organic compounds of hotel housekeepers: field measurements of exposure and health risks. *Indoor Air*. 2021;31:26–39. https://doi.org/10.1111/ina.12709.
9. Li X, Zhong Y, He W, et al. Co-exposure and health risks of parabens, bisphenols, triclosan, phthalate metabolites and hydroxyl polycyclic aromatic hydrocarbons based on simultaneous detection in urine samples from guangzhou, south China. *Environ Pollut*. 2021;272:115990. https://doi.org/10.1016/j.envpol.2020.115990.
10. Dos Anjos DBM, Ricardi AST, Fernandes CFB, et al. Severe acute toxic exposures in children and adolescents: case series. *Revista Paulista de Pediatria*. 2021;39. https://doi.org/10.1590/1984-0462/2021/39/2019262.
11. Franck MC, Monteiro MG, Limberger RP. Toxicology of suicide cases in the state of Rio Grande do Sul, Brazil, 2017 to 2019. *Revista Panamericana de Salud Pública*. 2021;45:45–e28. https://doi.org/10.26633/rpsp.2021.28.
12. De Souza JP, Nogueira GM, Mataqueiro MI, et al. Brain cholinesterase activity and the behavior of rats after perinatal exposure to dichlorvos. *Ciencia Rural*. 2006;36:501–508. https://doi.org/10.1590/s0103-84782006000200021.
13. Mishra A, Kumar J, Melo JS, et al. Progressive development in biosensors for detection of dichlorvos pesticide: a review. *J Environ Chem Eng*. 2021;9: 105067. https://doi.org/10.1016/j.jece.2021.105067.
14. Proskocil BJ, Bruun DA, Thompson CM, et al. Organophosphorus pesticides decrease M2 muscarinic receptor function in guinea pig airway nerves via indirect mechanisms. *PLOS ONE*. 2010;5:e10562. https://doi.org/10.1371/JOURNAL.PONE.0010562.
15. Costa L, Giordano G, Guizzetti M, et al. Neurotoxicity of pesticides: a brief review. *Front Biosci*. 2008;13:1240–1249. https://doi.org/10.2741/2758.
16. Jokanović M, Kosanović M. Neurotoxic effects in patients poisoned with organophosphorus pesticides. *Environ toxicol Pharmacol*. 2010;29:195–201. https://doi.org/10.1016/j.etap.2010.01.006.
17. Indudharan R, Win MN, Noor AR. Laryngeal paralysis in organophosphorous poisoning. *J Laryngol Otol*. 1998;112:81–82. https://doi.org/10.1017/s0022215100139969.
18. Jin Y, Jeong T, Lee J. Isolated bilateral vocal cord paralysis with intermediate syndrome after organophosphate poisoning. *Clin Toxicol*. 2008;46:482–484. https://doi.org/10.1080/15563650701704842.
19. Thompson JW, Stocks RM. Brief bilateral vocal cord paralysis after insecticide poisoning: a new variant of toxicity syndrome. *Arch Otolaryngol Head Neck Surg*. 1997;123:93–96. https://doi.org/10.1001/ARCHOTOL.1997.01900010103016.
20. de Silva H, Sanmuganathan P, Senanayake N. Isolated bilateral recurrent laryngeal nerve paralysis: a delayed complication of organophosphorus poisoning. *Hum Exp Toxicol*. 1994;13:171–173. https://doi.org/10.1177/096032719401300306.
21. Erdogan S, Handan Zeren E, Emre M, et al. Pulmonary effects of deltamethrin inhalation: an experimental study in rats. *Ecotoxicol Environ Saf*. 2006;63:318 323. https://doi.org/10.1016/j.ecoenv.2004.10.007.
22. Patel S, Pandey AK, Bajpayee M, et al. Cypermethrin-induced DNA damage in organs and tissues of the mouse: evidence from the comet assay. *Mutat Res*. 2006;607:176 183. https://doi.org/10.1016/j.mrgentox.2006.04.010.
23. Zhao H, Wang Y, Guo M, et al. Environmentally relevant concentration of cypermethrin or/and sulfamethoxazole induce neurotoxicity of grass carp: involvement of blood-brain barrier, oxidative stress and apoptosis. *Sci Total Environ*. 2021. https://doi.org/10.1016/j.scitotenv.2020.143054.
24. Yadav A, Tandon A, Seth B, et al. Cypermethrin impairs hippocampal neurogenesis and cognitive functions by altering neural fate decisions in the rat brain. *Mol Neurobiol*. 2021;58:263 280. https://doi.org/10.1007/s12035-020-02108-9.
25. Sheikh N, Javed S, Asmatullah Ahmad KR, et al. Histological changes in the lung and liver tissues in mice exposed to pyrethroid inhalation. *Walailak J Sci Technol*. 2014;11:843–849. https://doi.org/10.2004/wjst.v11i9.576.
26. Martínez MA, Lopez-Torres B, Rodríguez JL, et al. Toxicologic evidence of developmental neurotoxicity of Type II pyrethroids cyfluthrin and alpha-cypermethrin in SH-SY5Y cells. *Food Chem Toxicol*. 2020;137. https://doi.org/10.1016/j.fct.2020.111173.
27. Sayim F, Yavasoglu NÜK, Uyanikgil Y, et al. Neurotoxic effects of cypermethrin in Wistar rats: a haematological, biochemical and histopathological study. *J Health Sci*. 2005;51:300–307. https://doi.org/10.1248/jhs.51.300.
28. Kumar Singh A, Nath Tiwari M, Prakash O, et al. A current review of cypermethrin-induced neurotoxicity and nigrostriatal dopaminergic neurodegeneration. *Curr Neuropharmacol*. 2012;10:64–71. https://doi.org/10.2174/157015912799362779.
29. Ali H, El-Sayed NM, Khodeer DM, et al. Cellular Mechanism involved in cypermethrin induced neurotoxicity. *Records Pharm Biomed Sci*. 2020;4:32–39. https://doi.org/10.21608/rpbs.2019.17969.1043.
30. Crofton KM, Kehn LS, Gilbert ME. Vehicle and route dependent effects of a pyrethroid insecticide, deltamethrin, on motor function in the rat. *Neurotoxicol Teratol*. 1995;17:489–495. https://doi.org/10.1016/0892-0362(95)00008-F.
31. Ravula AR, Yenugu S. Pyrethroid based pesticides—chemical and biological aspects. *Crit Rev Toxicol*. 2021. https://doi.org/10.1080/10408444.2021.1879007.
32. Atiş S, Cömelekoğlu U, Coşkun B, et al. Electrophysiological and histopathological evaluation of respiratory tract, diaphragm, and phrenic nerve after dichlorvos inhalation in rats. *Inhal Toxicol*. 2002;14:199 215. https://doi.org/10.1080/08958370175340399.
33. Misik J, Pavlikova R, Cabal J, et al. Acute toxicity of some nerve agents and pesticides in rats. *Drug Chem Toxicol*. 2015;38:32–36. https://doi.org/10.3109/01480545.2014.900070.
34. Kamel F, Hoppin J. Association of pesticide exposure with neurologic dysfunction and disease. *Environ Health Perspect*. 2004;112:950–958. https://doi.org/10.1289/EHP.7135.
35. Naughton S, Hernandez C, Beck W, et al. Repeated exposures to diisopropylfluorophosphate result in structural disruptions of myelinated axons and persistent impairments of axonal transport in the brains of rats. *Toxicology*. 2018;406-407:92–103. https://doi.org/10.1016/J.TOX.2018.06.004.
36. Wang H, Liang Y, Sun Y, et al. Subchronic neurotoxicity of chlorpyrifos, carbaryl, and their combination in rats. *Environ toxicol*. 2014;29:1193–1200. https://doi.org/10.1002/TOX.21851.
37. De Campos D, Do Nascimento PS, Ellwanger JH, et al. Histological organization is similar in human vocal muscle and tongue - A study of muscles and nerves. *J Voice*. 2012;26:811.e19 811.e26. https://doi.org/10.1016/j.jvoice.2011.12.006.
38. De Campos D, Heck I, Jotz GP, et al. Degree of myelination (g-ratio) of the human recurrent laryngeal nerve. *Eur Arch Otorhinolaryngol*. 2014;271:1277–1281. https://doi.org/10.1007/s00405-013-2690-y.
39. Ilha J, Araujo RT, Malysz T, et al. Endurance and resistance exercise training programs elicit specific effects on sciatic nerve regeneration after experimental traumatic lesion in rats. *Neurorehabil Neural Repair*. 2008;22:355–366. https://doi.org/10.1177/1545968307313502.
40. Schenk HC, Haastert-Talini K, Jungnickel J, et al. Morphometric parameters of peripheral nerves in calves correlated with conduction velocity. *J Vet Intern Med*. 2014;28:646–655. https://doi.org/10.1111/jvim.12271.
41. Gillespie MJ, Stein RB. The relationship between axon diameter, myelin thickness and conduction velocity during atrophy of mammalian peripheral nerves. *Brain Res*. 1983;259:41–56. https://doi.org/10.1016/0006-8993(83)91065-X.

42. Dixon D, Herbert RA, Kissling GE, et al. Summary of chemically induced pulmonary lesions in the national toxicology program (NTP) toxicology and carcinogenesis studies. *Toxicol Pathol*. 2008;36:428–439. https://doi.org/10.1177/0192623308315360.
43. Yoshida T, Mimura M, Sakon N. Estimating household exposure to pyrethroids and the relative contribution of inhalation pathway in a sample of Japanese children. *Environ Sci Pollut Res*. 2021;28:19310–19324. https://doi.org/10.1007/s11356-020-12060-9.
44. Verdín-Betancourt FA, Figueroa M, Soto-Ramos AG, et al. Toxicokinetics of temephos after oral administration to adult male rats. *Arch Toxikol*. 2021;95:935–947. https://doi.org/10.1007/s00204-021-02975-8.
45. Ravula AR, Yenugu S. Effect of oral administration of a mixture of pyrethroids at doses relevant to human exposure on the general and male reproductive physiology in the rat. *Ecotoxicol Environ Saf*. 2021;208: 111714. https://doi.org/10.1016/j.ecoenv.2020.111714.
46. Keenan JJ, Vega H, Krieger RI. Potential exposure of children and adults to cypermethrin following use of indoor insecticide foggers. *J Environ Sci Health B*. 2009;44:538–545. https://doi.org/10.1080/03601230902997733.
47. Cavaliere MJ, Calore EE, Perez NM, et al. Organophosphate-induced myotoxicity. *Revista de Saude Publica*. 1996;30:267–272. https://doi.org/10.1590/s0034-89101996000300010.
48. Tsai YH, Lein PJ. Mechanisms of organophosphate neurotoxicity. *Curr Opin Toxicol*. 2021;26:49–60. https://doi.org/10.1016/J.COTOX.2021.04.002.
49. Farahat TM, Abdelrasoul GM, Amr MM, et al. Neurobehavioural effects among workers occupationally exposed to organophosphorous pesticides. *Occup Environ Med*. 2003;60:279–286. https://doi.org/10.1136/OEM.60.4.279.
50. Eells J, Dubocovich M. Pyrethroid insecticides evoke neurotransmitter release from rabbit striatal slices. *J Pharmacol Exp Ther*. 1988;246:514–521.
51. Kirby ML, Castagnoli K, Bloomquist JR. In vivo effects of deltamethrin on dopamine neurochemistry and the role of augmented neurotransmitter release. *Pestic Biochem Physiol*. 1999;65:160–168. https://doi.org/10.1006/pest.1999.2440.
52. Tan J, Liu Z, Wang R, et al. Identification of amino acid residues in the insect sodium channel critical for pyrethroid binding. *Mol Pharmacol*. 2005;67:513–522. https://doi.org/10.1124/mol.104.006205.
53. Narahashi T. Neuronal ion channels as the target site of insecticides. *Pharmacol Toxicol*. 1996;79:1–14. https://doi.org/10.1111/j.1600-0773.1996.tb00234.x.
54. Manna S, Bhattacharyya D, Mandal TK, et al. Neuropharmacological effects of alfa-cypermethrin in rats. *Indian J Pharmacol*. 2005;37:18–20. https://doi.org/10.4103/0253-7613.13849.
55. Elser BA, Kayali K, Dhakal R, et al. Combined maternal exposure to cypermethrin and stress affect embryonic brain and placental outcomes in mice. *Toxicol Sci*. 2020;175:182–196. https://doi.org/10.1093/toxsci/kfaa040.
56. Moser V. Animal models of chronic pesticide neurotoxicity. *Hum Exp Toxicol*. 2007;26:321–331. https://doi.org/10.1177/0960327106072395.
57. Rose RJ, Hartley WJ, Baker W. Laryngeal paralysis in Arabian foals associated with oral haloxon administration. *Equine Vet J Suppl*. 1981;13:171–176. https://doi.org/10.1111/J.2042-3306.1981.TB03477.X.
58. Wells G, Bradley R. Haloxon neurotoxicity in the pig. *Neuropathol Appl Neurobiol*. 1977;3:218–221.
59. Bouldin TW, Cavanagh JB. Organophosphorous neuropathy. I. A teased-fiber study of the spatio-temporal spread of axonal degeneraion. *Am J Pathol*. 1979;94:241–252.
60. Bouldin TW, Cavanagh JB. Organophosphorous neuropathy. II. A fine-structural study of the early stages of axonal degeneration. *Am J Pathol*. 1979;94:270.
61. Jayasinghe SS, Pathirana KD, Buckley NA. Effects of acute organophosphorus poisoning on function of peripheral nerves: a cohort study. *PLOS ONE*. 2012;7:e49405. https://doi.org/10.1371/JOURNAL.PONE.0049405.
62. Moretto A, Lotti M. Poisoning by organophosphorus insecticides and sensory neuropathy. *J Neurol Neurosurg Psychiatry*. 1998;64:463–468. https://doi.org/10.1136/JNNP.64.4.463.
63. Chomiak T, Hu B. What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. *PLoS ONE*. 2009;4:e7754. https://doi.org/10.1371/journal.pone.0007754.
64. Zhang J, Ren J, Liu Y, et al. Resveratrol regulates the recovery of rat sciatic nerve crush injury by promoting the autophagy of Schwann cells. *Life Sci*. 2020;256. https://doi.org/10.1016/j.lfs.2020.117959.
65. Ikeda M, Oka Y. The relationship between nerve conduction velocity and fiber morphology during peripheral nerve regeneration. *Brain Behav*. 2012;2:382–390. https://doi.org/10.1002/brb3.61.
66. Kim PD, Hayes A, Amin F, et al. Collagen nerve protector in rat sciatic nerve repair: a morphometric and histological analysis. *Microsurgery*. 2010;30:392–396. https://doi.org/10.1002/micr.20760.
67. Isaacs J, Friebe I, Malla S, et al. Neurotrophic effects of perfluorocarbon emulsion gel: a pilot study. *J Brachial Plex Peripher Nerve Inj*. 2011;6:11. https://doi.org/10.1186/1749-7221-6-11.
68. Smith DRR, Patterson RD. The interaction of glottal-pulse rate and vocal-tract length in judgements of speaker size, sex, and age. *J Acoust Soc Am*. 2005;118:3177–3186. https://doi.org/10.1121/1.2047107.
69. Hayashi M, Isozaki E, Oda M, et al. Loss of large myelinated nerve fibres of the recurrent laryngeal nerve in patients with multiple system atrophy and vocal cord palsy. *J Neurol Neurosurg Psychiatry*. 1997;62:234–238. https://doi.org/10.1136/jnnp.62.3.234.

11. ANEXOS

11.1. Parecer do Comitê de Ética da UFCSPA – Projeto 1

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UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) **PROTOCOLO Nº: 193/16**

2) DATA DO PARECER: 14/12/2016 Parecer 478/16

3) TÍTULO DO PROJETO:

Efeitos da exposição subcrônica inalatória da associação de Diclorvós e Cipermetrina na audição de ratos wistar.

4) PESQUISADOR RESPONSÁVEL:

Eliane Dallegrave

5) RESUMO DO PROJETO:

O projeto visa analisar efeitos sistêmicos e auditivos da exposição à associação de Diclorvós e Cipermetrina na audição de ratos Wistar. Este projeto se justifica uma vez que agricultores estão expostos a diversos agrotóxicos, e é sabido que a associação destes compostos pode potencializar os danos causados à saúde dentre estes a perda auditiva. No entanto, este tipo de avaliação somente foi realizada em trabalhadores também expostos ao ruído do maquinário, tornando necessária a realização de experimentos que avaliem em separado os efeitos dos compostos químicos em questão.

6) OBJETIVOS DO PROJETO:

Objetivo geral:
Avaliar os efeitos sistêmicos e auditivos da exposição à associação de Diclorvós e Cipermetrina na audição de ratos wistar.
Objetivos específicos: 1- Verificar a presença ou ausência de EOAPD nas frequências de 1,5, 2, 3, 4, 5, 6, 8, 10 e 12 kHz em ratos Wistar expostos a associação de Diclorvós e Cipermetrina. 2- Detectar possíveis mudanças na amplitude das EOAPD nas frequências de 1,5, 2, 3, 4, 5, 6, 8, 10 e 12 kHz em ratos Wistar expostos a associação de Diclorvós e Cipermetrina. 3- Avaliar otoscopicamente as estruturas da orelha externa e média em ratos Wistar expostos a associação de Diclorvós e Cipermetrina. 4- Descrever sinais clínicos decorrentes da exposição a associação de Diclorvós e Cipermetrina em ratos Wistar. 5 – Avaliar níveis de colinesterase plasmática em ratos Wistar expostos à associação de Diclorvós e Cipermetrina.

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6- Avaliar a massa relativa dos órgãos e a histologia pulmonar de ratos Wistar expostos à associação de Diclorvós e Cipermetrina.

7) FINALIDADE DO PROJETO: [ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[X] Adequado	[ ] Comentários
Introdução	[X] Adequada	[ ] Comentários
Objetivos	[X] Adequados	[ ] Comentários
Relevância e Justificativa	[X] Adequados	[ ] Comentários
Materiais e Métodos	[X] Adequados	[ ] Comentários
Cronograma para execução da pesquisa	[X] Adequado	[ ] Comentários
Orçamento e fonte financiadora	[X] Adequados	[ ] Comentários
Referências Bibliográficas	[X] Adequadas	[ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE: [X] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: B | C [ ] D [ ] E [ ]
Justifique: Segundo formulário novo GI1

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Espécie: *Ratus novergicus* - Wistar **Número Amostral:** 36

Redução Amostral: [ ] Sim [x] Não
Justifique:

Substituição de Metodologia: [ ] Sim [X] Não
Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [X] Não
Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Laboratório de Farmacologia sala 318 da UFCSPA.

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Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Janeiro de 2017 a Novembro de 2018.	*Ratus novergicus*	masculino	36

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início janeiro/2017 Data de Término novembro/2018

Comentários gerais sobre o projeto:

As questões levantadas foram respondidas adequadamente.

O presente projeto está aprovado pela CEUA-UFCSPA.

11.2. Parecer do Comitê de Ética da UFCSPA – Projeto 2

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

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UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 246/18

2) DATA DO PARECER: 12/12/18 **Parecer 597/18**

3) TÍTULO DO PROJETO:

Efeitos da exposição subcrônica inalatória a agrotóxicos na audição de ratos wistar

4) PESQUISADOR RESPONSÁVEL:

Eliane Dallegrave

5) RESUMO DO PROJETO:

Os agrotóxicos podem ocasionar distúrbios de audição em trabalhadores expostos ocupacionalmente na agricultura, produção animal, controle de vetores, controle de pragas em edificações comerciais ou mesmo domésticas. O estudo objetiva avaliar os efeitos da exposição subcrônica inalatória aos inseticidas organofosforado Diclorvós, piretroide Cipermetrina e associação de ambos, na audição de ratos. Para tal, serão utilizados 25 ratos wistar machos de 60 dias, alocados em 5 grupos: controle, controle positivo com lesão auditiva, Diclorvós, Cipermetrina e Diclorvós + Cipermetrina. A exposição será realizada durante 4 horas por dia, 5 vezes por semana, durante 6 semanas.

6) OBJETIVOS DO PROJETO:

Avaliar os efeitos da exposição subcrônica inalatória aos inseticidas organofosforado Diclorvós, piretroide Cipermetrina e associação de ambos, na audição de ratos.

7) FINALIDADE DO PROJETO: [X] Ensino [ ] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título [X] Adequado [ ] Comentários

Introdução [X] Adequada [ ] Comentários

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Objetivos	[X] Adequados	[ ] Comentários
Relevância e Justificativa	[X] Adequados	[ ] Comentários
Materiais e Métodos	[X] Adequados	[ ] Comentários
Cronograma para execução da pesquisa	[X] Adequado	[ ] Comentários
Orçamento e fonte financiadora	[X] Adequados	[ ] Comentários
Referências Bibliográficas	[X] Adequadas	[ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:
[X] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: B C [X] D [ ] E [ ]
Justifique:

Ensaios de toxicidade subcrônica apresentam desconfortos de intensidade moderada.

Espécie: Rato **Número Amostral:** 35

Redução Amostral: [ ] Sim [X] Não
Justifique:

Substituição de Metodologia: [ ] Sim [X] Não
Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [X] Não
Se achar necessário, justifique e sugira aprimoramentos da metodologia:

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Acomodação e manutenção dos animais: [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Biotério da UFCSPA

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
06/2019	Rato wistar	machos	35

12) RECOMENDAÇÃO: As pendências deverão ser respondidas em uma carta, indicando as páginas do projeto que foram alteradas (nova versão), assinadas pelo pesquisador responsável.

[X] Aprovado

[ ] Com Pendência

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☐ Não aprovado

Data de início 02/01/2018 Data de Término 02/01/2019

Comentários gerais sobre o projeto:

Projeto de pesquisa relevante e adequadamente elaborado, com todas questões éticas em uso de animais descritas.