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ABRAÃO DE JESUS BARBOSA

ALTERAÇÕES COMPORTAMENTAIS E FISIOLÓGICAS APÓS EXPOSIÇÃO CRÔNICA DE CLETODIM EM RATOS

SÃO CRISTÓVÃO – SE

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Fisiológicas da Universidade Federal de Sergipe como requisito à obtenção do grau de Mestre em Ciências Fisiológicas.

Orientador: Prof. Dr. José Ronaldo dos Santos

Coorientador: Prof. Dr. Auderlan M. de Gois

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EPÍGRAFE

"Quem come do fruto do
conhecimento é sempre expulso de algum
paraíso"
Melanie Klein

RESUMO

O cletodim (CL) é um herbicida seletivo do grupo químico oxima-ciclohexanodiona, cujo mecanismo de ação ocorre através da inibição da acetil coenzima A, sendo amplamente utilizado para o combate de plantas que apresentam resistência ao glifosato. Devido à escassez de estudos na literatura sobre os impactos desse composto na saúde humana ou animal, estudos que busquem avaliar sua toxicidade em organismos não-alvos se fazem necessários. O **objetivo** do presente trabalho foi avaliar as alterações comportamentais e fisiológicas após exposição repetida de CL em ratos. Como **materiais e métodos**, após obtida a aprovação da Comissão de Ética no Uso de Animais da Universidade Federal de Sergipe (CEUA/UFS) sob o protocolo de nº 5228100323, foram utilizados 30 ratos machos adultos com idade de 6-8 meses (350-500 g) da linhagem Wistar. Os animais foram divididos em 4 grupos com 7-8 animais por grupo, de acordo com a concentração do composto: controle (CTL-soro fisiológico 0,9%), CL nas concentrações de 0,06% (CL-0,06), 0,3% (CL-0,3) e 0,6% (CL-0,6). Foram realizadas 25 exposições por nebulização (uma a cada 48h, com duração de 10 minutos cada) com solução salina ou CL de acordo com o grupo experimental. Ao longo do experimento foram realizados testes comportamentais, a saber: catalepsia (a cada 48 h), campo aberto (CA - dia 0 e 38), reconhecimento de objeto novo (RO - dia 12), alternação espontânea (AE - dia 16) e teste de nado forçado (TNF – dia 25). Além disso, foram realizadas avaliações da massa corporal (a cada 4 dias), ingestão alimentar (dia 44), tolerância glicêmica (dia 48) e medida da massa dos órgãos e retirada dos encéfalos após perfusão (dia 50). Em seguida, os encéfalos foram processados para avaliação da imunorreatividade das proteínas Fator Neurotrófico Derivado do Cérebro (BDNF), c-Fos e Colina-O-Acetil-Transferase (ChAT). Nossos **resultados** demostraram que o grupo CL-0,06 apresentou menor tempo no teste de catalepsia e menor massa do baço; no grupo CL-0,6 foi observado menor tempo no centro no CA e prejuízo na memória operacional na AE, mas não no RO, aumento da variação corporal e menor pico glicêmico no teste de tolerância glicêmica; em todas as concentrações observamos menor consumo alimentar e maior número de eventos de imobilidade com maior tempo imóvel no TNF. Quanto às avaliações neuroquímicas, foi identificada uma menor imunorreatividade para BDNF e c-Fos em regiões hipocampais nos grupos CL-0,06 e CL-0,3, sem alterações quanto à expressão de ChAT em áreas colinérgicas. Como **conclusão**, nosso trabalho demostrou que a exposição ao CL de acordo a concentração foi capaz de promover comportamento do tipo ansioso e depressivo, déficit de memória, aumento da massa corporal, e menor atividade hipocampal.

Palavras-chave: Agentes neurotóxicos; Produtos químicos agrícolas; Poluentes; Herbicidas.

ABSTRACT

Clethodim (CL) is a selective herbicide belonging to the oxime-cyclohexanedione chemical group, whose mechanism of action involves the inhibition of acetyl coenzyme A. It is widely used to combat plants that are resistant to glyphosate. Due to the scarcity of studies in the literature on the impacts of this compound on human or animal health, studies seeking to assess its toxicity in non-target organisms are necessary. The objective of this study was to evaluate behavioral and physiological changes after repeated exposure to CL in rats. As materials and methods, after obtaining approval from the Animal Use Ethics Committee of the Federal University of Sergipe (CEUA/UFS) under protocol no. 5228100323, 30 adult male rats aged 6-8 months (350-500 g) of the Wistar strain were used. The animals were divided into four groups with seven to eight animals per group, according to the concentration of the compound: control (CTL-0.9% saline solution), CL at concentrations of 0.06% (CL-0.06), 0.3% (CL-0.3), and 0.6% (CL-0.6). Twenty-five nebulization exposures were performed (one every 48 hours, lasting 10 minutes each) with saline solution or CL according to the experimental group. Throughout the experiment, behavioral tests were performed, namely: catalepsy (every 48 hours), open field (OF - days 0 and 38), novel object recognition (NOR - day 12), spontaneous alternation (SA - day 16), and forced swim test (FST - day 25). In addition, assessments of body mass (every 4 days), food intake (day 44), glycemic tolerance (day 48), and organ mass measurement and brain removal after perfusion (day 50) were performed. The brains were then processed to assess the immunoreactivity of Brain-Derived Neurotrophic Factor (BDNF), c-Fos, and Choline-O-Acetyl-Transferase (ChAT) proteins. Our results showed that the CL-0.06 group had a shorter time in the catalepsy test and lower spleen mass; in the CL-0.6 group, we observed a shorter time in the center in the CA and impairment in working memory in the AE, but not in the RO, increased body variation, and lower glycemic peak in the glucose tolerance test; in all concentrations, we observed lower food consumption and a higher number of immobility events with longer immobility time in the TNF. Regarding neurochemical assessments, lower immunoreactivity for BDNF and c-Fos was identified in hippocampal regions in the CL-0.06 and CL-0.3 groups, with no changes in ChAT expression in cholinergic areas. In conclusion, our study demonstrated that exposure to CL according to concentration was capable of promoting anxiety- and depression-like behavior, memory deficits, increased body mass, and decreased hippocampal activity.

Keywords: Neurotoxic agents; Agricultural chemicals; Pollutants; Herbicides.

RESUMO VOLTADO PARA A SOCIEDADE

Os agrotóxicos, pesticidas ou venenos, são produtos químicos utilizados para plantas ou animais que causem prejuízos para a lavoura. De modo geral, as pessoas utilizam os agrotóxicos sem utilizar de equipamentos de proteção individual (EPI), como máscaras, botas e roupas adequadas, além de usar quantidades maiores que as recomendadas no rótulo. Quando usado de forma errada, os venenos podem contaminar os trabalhadores e causar diversos problemas de saúde. No comércio há diversos agrotóxicos disponíveis para uso, um dos mais conhecidos é o Roundup (herbicida glifosato), também conhecido por mata tudo que é utilizado principalmente no plantio de transgênicos e que consegue destruir diversas espécies de plantas. De forma semelhante, encontra-se o herbicida Select, também conhecido como cletodim que elimina diversas espécies de gramíneas, principalmente as que o glifosato não consegue eliminar. Nos últimos anos, o uso do cletodim tem aumentado cada vez mais e, isso tem gerado preocupações sobre quais são os efeitos desse composto na saúde dos animais e para o meio ambiente, pois seus efeitos são pouco conhecidos. Diante disso, tivemos como **objetivo** avaliar se ratos expostos no longo prazo ao cletodim através de inalação apresentariam prejuízos motores, de memória, emocionais e em substâncias químicas no cérebro dos ratos. Como **materiais e métodos**, após obtida a aprovação da Comissão de Ética no Uso de Animais da Universidade Federal de Sergipe (CEUA/UFS) sob o protocolo de nº 5228100323, os animais foram expostos ao composto através de um aparelho nebulizador pelo ar inalado, para que pudéssemos fazer de forma parecida com o que acontece com os trabalhadores da zona rural, que ao pulverizar a lavoura, parte daquele líquido entra em contato direto com a pele e outra parte é inalado. Utilizamos três concentrações diferentes para expor os ratos, a primeira foi igual a concentração usada pelos agricultores no campo, a segunda concentração foi cinco vezes mais alta e a terceira dez vezes mais concentrada para o terceiro grupo, além de termos um grupo controle que só foi exposto ao soro fisiológico. Os nossos **resultados** demonstraram que a concentração em que os agricultores utilizam causou redução da massa do baço e menor atividade dos neurônios em uma área responsável pela formação de memória nos ratos. No grupo exposto à concentração dez vezes mais concentrada, foi observado prejuízos na formação da memória de curto prazo, comportamento ansioso e um atraso para ter um nível mais elevado de glicose na corrente sanguínea, refletindo em alterações na absorção da glicose e o seu envio para a corrente sanguínea. Além disso, observamos comportamento desmotivado dos animais no teste de nado forçado, pois todos os que foram expostos ao cletodim tiveram maior tempo parado. Por fim, através deste estudo, podemos ter como **conclusão** que o cletodim, mesmo na concentração recomendada causa prejuízos aos animais expostos. Esses achados reforçam a importância do uso de EPI pela população, assim como, incentivar os agricultores a utilizarem a concentração recomendada pelo fabricante, pois percebemos maior número de alterações na concentração mais elevada.

Palavras-chave: Agrotóxicos; Agente Neurotóxico; Contaminantes Ambientais.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ACC – Acetil-coenzima A carboxilase

ACh – Acetilcolina

AChE – Acetilcolinesterase

AE – Alternação espontânea

BDNF – Fator neurotrófico derivado do cérebro

CA – Campo aberto

CA1 – Corno de Amon 1

CA3 – Corno de Amon 3

ChAT – Colina acetiltransferase

CL – Cletodim

CPm – Córtex pré-frontal medial

CTL – Controle

DDT - 1,1,1-tricloro-2,2-bis (4-clorofenil) etano

DG - Dentate gyrus

EPIs – Equipamentos de proteção individual

FST – Forced swim test

GABA – Ácido gama aminobutírico

GD – Giro denteado

IC – Insular cortex

mPFC – Medial prefrontal cortex

MS – Medial septum

NBM – Núcleo basal de Meynert

nbM – Nucleus basalis of Meynert

NOR – Novel object recognition

OC – Oxima ciclohexanodiona

OD – Optical densitometry

OF – Campo aberto

OPs – Organofosforados

RO – Reconhecimento de objeto novo

SA – Spontaneous alternation

SNC – Sistema nervoso central

SNP – Sistema nervoso periférico

TNF – Teste de nado forçado

SUMÁRIO

1. INTRODUÇÃO

No ano de 2023 foram comercializados aproximadamente 3.7 milhões de toneladas de agrotóxicos, fazendo parte desse número os herbicidas, inseticidas e fungicidas (FAOSTAT, 2023). Essa utilização tem aumentado cada vez mais devido a maior facilidade, menores custos e pelo maior controle de pragas (ervas daninhas ou animais) que acometem a produção agrícola, melhorando a produtividade do plantio (Zhou, Li e Achal, 2025).

Esses compostos possuem princípios ativos com a capacidade de causar a mortalidade de um organismo-alvo, mediante o seu mecanismo de ação e sua seletividade (Ahmad *et al.*, 2024; Akashe, Pawade e Nikam, 2018; Richardson *et al.*, 2019). Além disso, essas substâncias podem se acumular nos organismos causando a bioacumulação, através da ingestão direta do composto ou com alimentos contaminados, inalação, contato com a pele, poros, raízes ou folhas (Chormare e Kumar, 2022). Os organismos contaminados podem repassar a sua contaminação ao servir de alimento para outros consumidores ao longo da cadeia alimentar, causando a biomagnificação (Franke *et al.*, 1994; Tison *et al.*, 2024). Alguns dos fatores que contribuem para a dispersão desses contaminantes são a dispersão pelo ar, adsorção, lixiviação, volatilização e o escoamento (Sánchez-Bayo, 2021).

Essa contaminação ao ser repassada pode atingir espécies não-alvos, afetando a sua reprodução, o tamanho, a sobrevivência e causando citotoxicidade em diversos organismos (Wan *et al.*, 2025). Muitas vezes, o contato com esses contaminantes ambientais não causará de imediato a manifestação dos sintomas visíveis, porém podendo afetar de forma silenciosa os sistemas reprodutor, endócrino, imune e nervoso dos animais (Kalyabina *et al.*, 2021; Ray e Shaju, 2023).

Na agricultura, os trabalhadores podem se contaminar com essas substâncias tóxicas de diversas formas, desde o ato de preparar a calda até realizar a sua pulverização, dada a ausência do uso de equipamentos de proteção individual (EPIs) de boa qualidade (corretos) (Lari *et al.*, 2022). Tais contaminações podem ocorrer por diferentes vias, tais como inalatória, oral e dérmica (Tudi *et al.*, 2022). De acordo com o tempo de exposição, os sintomas de intoxicação pelos agrotóxicos podem se manifestar de forma diferente. Exposições agudas podem causar principalmente erupções na pele, náuseas, vômitos, tonturas, dor de cabeça e fadiga (Pengpan *et al.*, 2024), enquanto a crônica pode contribuir para o desenvolvimento de algumas doenças como cânceres, autoimunes, neurológicas (Parkinson e Alzheimer) e causar perturbações no sistema endócrino (Richardson et al., 2019; Shekhar et al., 2024; Ahmad et al., 2024).

De acordo com o mecanismo de ação e especificidade de cada pesticida, estes são classificados em diferentes grupos, sendo o grupo organofosforado (OPs) o mais utilizado e

estudado, principalmente por ter o herbicida glifosato como o agrotóxico mais utilizado no mundo, o qual possui mais de 10.000 artigos publicados, abordando sobre sua eficiência e toxicidade em organismos-alvos e não-alvos (Akashe; Pawade; Nikam, 2018; Castilhos et al., 2020). Entretanto, nos últimos anos outros herbicidas estão ganhando destaque pelo aumento de sua utilização, carecendo, porém, de informações sobre a sua toxicidade em organismos não-alvos e se eles acarretam em prejuízos para a saúde humana.

Nesse cenário, encontra-se o cletodim (CL), que vem sendo amplamente utilizado no controle de plantas resistentes ao glifosato (Agostinetto et al., 2022; Merotto et al., 2022). Nos últimos anos esse composto apresentou um aumento de 2300% da sua utilização nas lavouras (Merotto *et al.*, 2022). No entanto, pouco se conhece sobre os efeitos fisiológicos desse composto sobre organismos não-alvos. De acordo com a literatura, sabe-se que a exposição ao CL em ratos pode causar alterações nos testículos (Dcunha *et al.*, 2023), redução da massa corporal (Abuzeid et al., 2021), hepatotoxicidade (Ergenekon, Erman e Şimşek Özek, 2024) e, em peixes-zebra, neurotoxicidade acompanhada de anormalidades morfofisiológicas (Wang *et al.*, 2019; Xiong *et al.*, 2019).

Diante disso, o presente estudo objetivou avaliar as alterações comportamentais e fisiológicas após exposição repetida de CL em ratos.

2. REVISÃO DA LITERATURA

2.1 AGROTÓXICOS

Agrotóxicos, pesticidas ou defensivos agrícolas, são produtos químicos ou misturas de substâncias utilizadas para o combate ou eliminação de organismos-alvo que acometem a produção agrícola (INCA, 2021). Esses compostos não são utilizados somente no setor agrícola, pois grande parte da população da zona rural ou urbana os empregam no âmbito doméstico para o controle de insetos, a exemplo, os inseticidas. Quanto à classificação desses químicos, de forma geral, eles podem ser classificados de acordo com a toxicidade, grupo químico, mecanismo de ação e organismo-alvo (Akashe; Pawade; Nikam, 2018; Richardson et al., 2019). Sobre a toxicidade, quanto menor o número da categoria mais tóxico é o agrotóxico caso seja ingerido, inalado ou entre em contato com a pele, conforme mostrado na figura 1 abaixo.

	CATEGORIA 1	CATEGORIA 2	CATEGORIA 3	CATEGORIA 4	CATEGORIA 5	NÃO CLASSIFICADO
	EXTREMAMENTE TÓXICO	ALTAMENTE TÓXICO	MODERADAMENTE TÓXICO	POUCO TÓXICO	IMPROVÁVEL DE CAUSAR DANO AGUDO	NÃO CLASSIFICADO
PICTOGRAMA					Sem símbolo	Sem símbolo
PALAVRA DE ADVERTÊNCIA	PERIGO	PERIGO	PERIGO	CUIDADO	CUIDADO	Sem advertência
CLASSE DE PERIGO						
Oral	Fatal se ingerido	Fatal se ingerido	Tóxico se ingerido	Nocivo se ingerido	Pode ser perigoso se ingerido	-
Dérmica	Fatal em contato com a pele	Fatal em contato com a pele	Tóxico em contato com a pele	Nocivo em contato com a pele	Poder ser perigoso em contato com a pele	-
Inalatória	Fatal se inalado	Fatal se inalado	Tóxico se inalado	Nocivo se inalado	Pode ser perigoso se inalado	-
COR DA FAIXA	Vermelho PMS Red 199 C	Vermelho PMS Red 199 C	Amarelo PMS Yellow C	Azul PMS Blue 293 C	Azul PMS Blue 293 C	Verde PMS Green 347 C

Figura 1: Classificação quanto a toxicidade dos agrotóxicos. Fonte: Adaptado de MAPA (2022).

A classificação de acordo com o grupo químico é baseada nos arranjos químicos dos princípios ativos presentes nas formulações desses compostos, sendo os principais grupos os organofosforados (OPs), organoclorados, organofluorados, carbamatos, triazinas, piretróides, neocotinoides, inibidores do complexo mitocondrial I, fenilpirazóis e sulfonilureias (Akashe; Pawade; Nikam, 2018; Jayaraj; Megha; Sreedev, 2016; Liu et al., 2023; Ogawa et al., 2020; Singh et al., 2021).

Os OPs, são conhecidos por serem derivados dos ácidos fosfórico, fosfônico ou fosfínico. Uma característica marcante desse grupo é o fato de se ligarem de forma irreversível à enzima acetilcolinesterase (AChE), fazendo com que ocorra o acúmulo de acetilcolina (ACh) na fenda sináptica, causando o excesso desse neurotransmissor (Peter, Sudarsan e Moran,

2014). Os primeiros produtos OPs foram produzidos pelo químico alemão Gerhard Schrader no século XX para fins bélicos. Posteriormente suas propriedades como inseticida e herbicida começaram a ser investigadas, dando origem a vários princípios ativos conhecidos até os dias atuais, como glifosato, clorpirifós, malatião, paratião e diazião (Terry, 2012; Vale; Lotti, 2015).

Quanto aos organoclorados, conhecidos pela presença da molécula de cloro em sua composição, o mais conhecido desse grupo é o etano clorado 1,1,1-tricloro-2,2-bis (4-clorofenil) (DDT), com uma mistura de isômeros sendo presente em sua formulação, como o *p,p'* – DDT e o *o,p'* – DDT, desempenhando propriedades inseticidas e de desreguladores endócrinos (Interdonato *et al.*, 2023). Outros bastante utilizados são o metoxicloro (2,2 – bis (p-metoxifenil) – 1,1,1 - tricloroetano), análogo *p,p'* – dimetoxi do *p,p'* – DDT, dieldrin, aldrin e lindano (Jayaraj, Megha e Sreedev, 2016; Shukla *et al.*, 2014). Além das propriedades inseticidas utilizadas na agricultura e em âmbito doméstico, eles foram bastante empregados em formulações de shampoos para o combate de piolhos e fungos (Costa, 2015).

O grupo dos carbamatos é conhecido por ser derivado do ácido N-metilcarbâmico, compostos pouco solúveis em água, porém bastante solúveis em solventes orgânicos polares. Semelhantes aos OPs, eles desempenham o seu mecanismo de ação através da inibição reversível da AChE, mas com menor tempo de inibição que os OPs, sendo divididos em dois grandes grupos: inseticidas anti-colinérgicos (miticidas como carbaril, aldicarbe, metomil e o carbofurão) e os tiocarbamatos, subdivididos de acordo com as suas propriedades em fungicidas (tirame, manebe e o zinebe) e herbicidas (metame e o pebulate) (Alvares, 1992; Vale e Lotti, 2015).

As triazinas são um grupo de herbicidas que apresentam estruturas químicas semelhantes entre si (clorotriazinas, atrazina, propazina e simazina), diferenciando-se em apenas um substituinte, a s-triazina 4- e 6-N-alquil (USEPA, 2018). Esse grupo é considerado moderadamente persistente no ambiente e com degradação lenta, acarretando na toxicidade dos organismos não alvos, promovendo a inibição da cadeia respiratória mitocondrial em animais (Abass, Pelkonen e Rautio, 2021; Lim *et al.*, 2009).

Os neocotinoides (imidacloprido, nitenpiram, tiametoxam, tiacloprido, acetamprimido, clotianidina e dinotefurano) são caracterizados por serem utilizados como inseticidas para o combate de insetos que danificam as folhas das plantações e durante o armazenamento de sementes (Tomizawa e Casida, 2005; Wang *et al.*, 2018). Esse grupo apresenta menor toxicidade do que os OPs, devido a suposta seletividade pelos receptores colinérgicos e nicotínicos dos invertebrados, reduzindo a toxicidade para os vertebrados (Crosby *et al.*, 2015; Tomizawa e Casida, 2003).

Os inseticidas do grupo dos piretróides (deltametrina, cipermetrina, fenpropatrina, fenvalerato, bifentrina, permetrina, λ-cialotrina e ciflutrina) são compostos derivados da planta *Chrysanthemum cinerariaefolium*. Essas substâncias são bastante utilizadas no setor agrícola e de uso doméstico, apresentando alta toxicidade e especificidade para insetos e baixa para mamíferos (Elser, Hing e Stevens, 2022). Esse grupo tem como principal alvo os canais de sódio dependente de voltagem, alterando sua a cinética e favorecendo o aumento do influxo de sódio, causando despolarizações contínuas (Lu *et al.*, 2019; Matsuo, 2019).

No grupo dos inseticidas fenilpirazóis, o fipronil é o mais conhecido e utilizado. Esse grupo causa toxicidade e mortalidade dos insetos por meio da interferência da passagem dos íons através do canal cloreto 1 regulado pelo ácido gama-aminobutírico (GABA), promovendo alterações no funcionamento do sistema nervoso central (SNC), causando hiperexcitação, convulsões e paralisia (Singh *et al.*, 2021). Em vertebrados, esses compostos atuam de forma semelhante, entretanto não acarretando em alta toxicidade, pois são mais tóxicos para os invertebrados (Tingle et al., 2003).

Os herbicidas sulfonilureias (metsulfurão-meti, bensulfurão-metil, sulfometurão-metil, tifensulfurão-metil, tribenurão-metil, etametsulfurão-metil e clorimurão-etil) apresentam a estrutura molecular formada basicamente por um grupo arila, uma ponte de sulfonilureia e um heterociclo. Esse grupo tem como alvo a enzima aceto-hidroxiácido sintase (AHAS, EC 2.2.1.6) que é responsável por realizar a síntese de aminoácidos de cadeia ramificada em plantas, fungos e bactérias, com baixa toxicidade para mamíferos (Chaleff e Mauvais, 1984; Liu *et al.*, 2023).

Um grupo bastante diverso são os organofluorados (trifluralina, sulfluramida, flursulamida, nicofluprole e flubeneteram), que possuem o flúor como a substância marcante desse grupo, sendo o menor elemento e o mais eletronegativo da tabela periódica, possuindo diferentes formulações comerciais de acordo com a seleção do organismo-alvo (inseticida, herbicida, fungicida e acaricida) (Donley *et al.*, 2024; Ogawa *et al.*, 2020).

2.2 HERBICIDAS E INSETICIDAS

Os agrotóxicos possuem várias classes de acordo com o alvo: fungicidas, acaricidas, avicidas, formicidas, raticidas, inseticidas e herbicidas. Essas duas últimas classes são as mais utilizadas em todo o mundo, sendo conhecidas por desempenharem a sua toxicidade nos organismos-alvo e não-alvo, principalmente através da inibição da AChE e butirilcolinesterase em insetos e mamíferos (Čadež *et al.*, 2021; Thapa, Lv e Xu, 2017). Essa inibição acarreta em grandes prejuízos para os seres vivos, principalmente nos sistemas nervoso periférico (SNP) e central (SNC). A depender da concentração e o tempo de exposição, estes compostos podem

acarretar sobrecarga do sistema colinérgico, produzindo vários radicais livres, aumentando o estrese oxidativo e causando efeitos neurotóxicos, como o bloqueio dos receptores nicotínicos e muscarínicos, que podem resultar em mortalidade de animais (Naughton e Terry, 2018).

A enzima AChE é encontrada tanto no SNP como no SNC, sendo responsável por realizar a hidrólise do neurotransmissor ACh em ácido acético e a colina, para que não ocorra o acúmulo desse neurotransmissor na fenda sináptica, com consequente melhoria no reaproveitamento energético (Thapa, Lv e Xu, 2017). O acúmulo pode causar despolarizações contínuas ou inibição dos potenciais de ações neuronais, pois o desbalanço dos seus níveis pode alterar os níveis de outros neurotransmissores, como o GABA, glutamato e dopamina (Faro *et al.*, 2022; Martínez *et al.*, 2018; Petreski *et al.*, 2020). A colina é recaptada pelo neurônio pré-sináptico através de um cotransportador de sódio e colina, para que assim a enzima colina acetiltransferase (ChAT) realize a síntese da ACh a partir da colina recaptada em combinação com a acetil-coenzima A (acetil-CoA) e fique armazenada até o momento da exocitose pelo neurônio pré-sináptico (Fonnum; Malthe-Sørenssen, 1972; Mautner, 1977; Pilar; Vaca, 1979).

A inibição da AChE pelos pesticidas causa vários sintomas em humanos, tais como salivação, desconforto gastrointestinal, aumento da micção, bradicardia, lacrimejamento, miose, alterações no sistema respiratório, perda da consciência, hipertensão e até a morte (Petreski *et al.*, 2020; Yurumez *et al.*, 2007). Entretanto, os herbicidas e os inseticidas não causam somente essas alterações direcionadas para a inibição dessa enzima. A literatura mostra que eles estão associados ao surgimento de doenças autoimunes, cânceres, distúrbios mentais e comportamentais (ansiedade e depressão), doenças neurodegenerativas (Parkinson e Alzheimer), ideações suicidas, alterações no sistema endócrino, entre outros (Corralo *et al.*, 2016; Gonzaga, Baldo e Caldeira, 2021; Medeiros, Acayaba e Montagner, 2021; Salcedo-Arteaga e Schuler-Faccini, 2022).

Além disso, ao atravessarem a barreira hematoencefálica podem contribuir para a redução de algumas neurotrofinas no encéfalo, envolvidas com a plasticidade neuronal, como o fator neurotrófico derivado do cérebro (BDNF) (Li et al., 2019; Mahmoud et al., 2019), o qual está envolvido desde a comunicação neuronal, neuroproteção até a formação de memórias (Bekinschtein, Cammarota e Medina, 2014; Chapleau *et al.*, 2009; Colucci-D'amato, Speranza e Volpicelli, 2020). Níveis reduzidos dessa neurotrofina pela exposição aos pesticidas são associados com efeitos neurotóxicos, como o aumento do estresse oxidativo, neuroinflamação, comportamento do tipo depressivo e déficit de memória (Dorri *et al.*, 2015; Li *et al.*, 2019; Ribeiro *et al.*, 2021).

Ademais, já é sabido que o BDNF possui a capacidade de promover despolarizações e a deflagração de potenciais de ação em neurônios em diversas regiões encefálicas, através da ativação dos receptores TrkB da tirosina quinase (Kafitz *et al.*, 1999; Scharfman *et al.*, 2005). A ativação desses receptores nos neurônios pré e pós-sinápticos sinalizam o BDNF a se ligar de forma retrograda ou anterógrada nos terminais pré-sinápticos (Leal; Comprido; Duarte, 2014; Yano et al., 2006). Assim, essa neurotrofina irá promover o controle ou a modulação da transmissão sináptica durante a liberação de neurotransmissores, visto que a ausência do receptor TrkB interfere na maturação sináptica e no número de vesículas sinápticas liberadas (Martínez *et al.*, 1998).

Alterações nas neurotrofinas pela exposição aos pesticidas podem comprometer a atividade neuronal, promovendo a redução da tradução do gene *c-fos* na proteína c-Fos (Imamura *et al.*, 2005; Liu *et al.*, 2021; Varma *et al.*, 2024) ou seu aumento (Kubo et al., 2022; Rodriguez; Giordano, 2017; Wu; Aiguo, 2003). Em condições basais, a expressão desse gene encontra-se baixa, porém após estímulo o nível de sua expressão é aumentado após 20 a 90 minutos, enquanto que o pico para a tradução ocorre entre 90 e 120 minutos após o estímulo (Lara Aparicio *et al.*, 2022; Sagar, Sharp e Curran, 1988).

A proteína c-Fos possui participação ativa durante os processos de formação de memória, atuando na manutenção do potencial de longa duração, além de auxiliar na modulação da depressão de longa duração (Gandolfi *et al.*, 2017; Giese, 2012; Khan *et al.*, 2025). Em modelos animais, é demonstrado que a exposição a agrotóxicos, como o paraquat, maneb e a permetrina, podem acarretar em redução de c-Fos juntamente com a proteína BDNF, acarretando em um menor influxo de cálcio e déficit de memória (Imamura *et al.*, 2005; Liu *et al.*, 2021). Porém, a depender do mecanismo de ação do pesticida, este pode induzir aumento de sua expressão, causando citotoxicidade pela liberação incessante de glutamato (Betancourt, Filipov e Carr, 2007; Wu e Liu, 2003).

Estudos apontam o papel neurotóxico dos agrotóxicos nos sistemas biológicos, como a Rotenona, Paraquat, Mancozebe, Malatião e DDT (Costa, 2015; Mohammadzadeh et al., 2018; Candia; Zolezzi; Inestrosa., 2019; Wu; Johnson, 2011). Além desses compostos, outros têm se destacado como o glifosato, atrazina e a deltametrina, devido aos seus efeitos lesivos no sistema nervoso de ratos (Li *et al.*, 2018; Lu *et al.*, 2019; Luna *et al.*, 2021; Moser *et al.*, 2012). Entretanto, outros herbicidas são bastante utilizados e carecem de informações sobre sua toxicidade, como os compostos do grupo químico oxima-ciclohexanodiona, que são o cletodim, aloxidim, butroxidim, cycloxidim, profoxidim, sethoxydim, lepraloxydim e tralkoxydim (Sandín-España *et al.*, 2012; Wang *et al.*, 2019).

2.3 OXIMA CICLOHEXANODIONA

O grupo oxima ciclohexanodiona, são uma classe de herbicidas pós-emergentes conhecido por serem compostos inibidores da acetil-coenzima A Carboxilase (ACC) em plantas de folhas estreitas (monocotiledôneas), principalmente as gramíneas anuais e perenes (Rendina e Felts, 1988). Esse grupo apresenta especificidade para o combate de ervas daninhas das lavouras de folhas largas (soja, beterraba, batata, melancia, entre outras), devido a sua especificidade em eliminar as plantas de folhas estreitas (Rajak *et al.*, 2023; Sandín-España *et al.*, 2012). Essa capacidade é atribuída as mudanças estruturais presentes na ACC das plantas de folhas estreitas, pois são menores e diferentes molecularmente, quando comparada com a ACC das de folhas largas, mas que desempenham a mesma função durante a biossíntese de novos ácidos graxos constituintes de membrana (Incledon e Hall, 1997).

De acordo com os boletins anuais de produção, importação, exportação e vendas de agrotóxicos no Brasil, segundo o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) do ano de 2019 a 2023, um dos compostos pertencentes ao grupo químico OC mais vendido foi o cletodim (CL) (IBAMA, 2025).

Na América do Sul, nos últimos anos (2009 – 2019), o uso do CL aumentou de forma exponencial, apresentando aumento de 2300% (Merotto *et al.*, 2022). Esse aumento pode estar relacionado ao fato de que diversas plantas da família das poáceas (gramíneas) apresentarem resistência ao glifosato (Heap e Duke, 2018). Diante disso, faz-se necessária a utilização de outros herbicidas, destacando os herbicidas seletivos inibidores da ACC para combater essas ervas daninhas, resultando no aumento do uso de CL (Merotto *et al.*, 2022; Sandín-España *et al.*, 2012).

2.4 CLETODIM

O (+/-)-2-[(E)-1-[(E)3-chloroallyloxymino]propyl]-5-[2--(ethylthio)propyl]-3-hidroxy-2-cyclohexen-1-one ($C_{17}H_{26}ClNO_3S$, figura 2), é um herbicida quiral sistêmico pós – emergente, com capacidade seletiva, tendo como alvo principal as gramíneas anuais e perenes (Dias Alves et al., 2021; Pedrollo et al., 2015; Vargas et al., 2006) e possui dois enantiômeros conhecidos: o R-(-)-cletodim e S-(+)-cletodim (Li *et al.*, 2025). O mecanismo de ação pelo qual o CL induz a mortalidade dos seus alvos ocorre por meio da inibição da ACC nas plantas de folhas estreitas, mas sem alterações nas de folhas largas, devido as mudanças estruturais e conformacionais da ACC (Fleck *et al.*, 2008; Incledon e Hall, 1997).

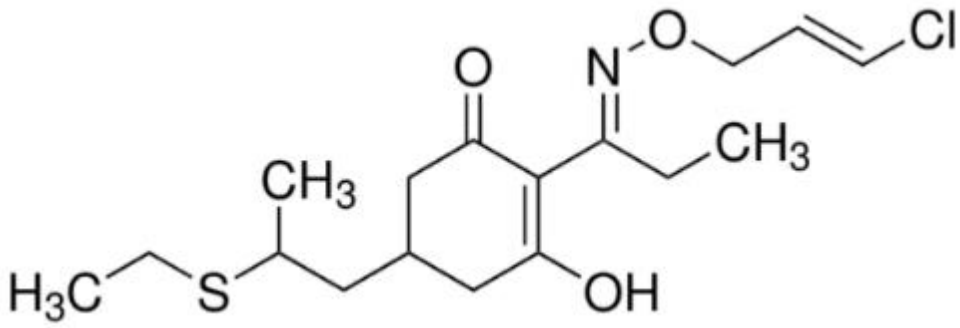

Figura 2: Molécula do cletodim. Fonte: Sigma Aldrich.

A degradação do CL no meio ambiente ocorre principalmente através da fotodegradação, que ocorre em menos de 24 h, gerando principalmente os metabólitos sulfóxido de CL, ácido trans-3-cloroacrílico e ácido 2-[3-cloroaliloximino] butanoico (EFSA, 2011). Além desses metabólitos, o CL no total possui 9 fotoprodutos: a forma isomérica de CL (C1), derivado de decloroaliloxi (C2), sulfóxidos de CL (C5 – C6), isômeros Z (C3 – C4), sulfóxidos do derivado decloroaliloxi (C7 – C9) e uma cetona decloroaliloxi (C9). Alguns desses fotoprodutos podem ser preocupantes para a saúde, pois são fotoquimicamente estáveis e podem permanecer maior tempo no meio aquoso do que a própria molécula de CL (Villaverde *et al.*, 2018); no entanto, a literatura não apresenta estudos utilizando modelos animais para avaliar a toxicidade dos seus metabólitos, salvo alguns poucos estudos com o CL em peixes – zebra e ratos (Dcunha et al., 2023; Abuzeid et al., 2021; Wang et al., 2019).

De acordo com os estudos disponíveis a respeito do CL, a maioria destes está relacionada à sua eficiência no combate de capins e gramíneas resistentes ao glifosato (Fleck *et al.*, 2008; Grichar *et al.*, 2002), utilizado em associação com outros herbicidas (Pedrollo et al., 2015; Carvalho et al., 2019) ou sobre a sua fitotoxicidade na germinação de sementes (Santos *et al.*, 2023). Entretanto, quanto aos efeitos tóxicos e neurotóxicos em humanos e demais animais, ela se encontra escassa (Wang *et al*., 2019; Dcunha *et al*., 2023; Abuzeid *et al*., 2021).

A respeito sobre a toxicidade do CL, os trabalhos de Wang *et al*., (2019) e Xiong *et al*., (2019) mostraram que ao expor os peixes – zebra, os animais apresentaram diversos sinais de toxicidade, como deformações na cabeça, olhos e cauda, redução da atividade da AChE, estresse oxidativo, mortalidade de acordo com a dose, imunotoxicidade, entre outros.

Quanto ao nível de efeito adverso não observado (NOAEL) para o CL em mamíferos, ele varia de 16 a 133,37 mg/kg^{-1} (EFSA, 2011). No trabalho de Dcunha *et al*., (2023), foram utilizados camundongos suíços machos albinos, tratados com CL diariamente durante dez dias via gavagem, nas doses de 50, 100 e 200 mg/kg^{-1} de massa corporal, e como resultado, com o CL na dose de 200 m/kg^{-1} sendo capaz de reduzir a capacidade de síntese de testosterona nas

células de Leydig, reduzir o número de células germinativas e também da motilidade dos espermatozoides.

No trabalho de Abuzeid *et al*., (2021) foi mostrado que a administração por gavagem de uma formulação comercial de CL (886 e 1773 mg/kg^{-1}) ou CL puro (163 e 326 mg/kg^{-1}) em ratos machos albinos, diariamente durante 28 dias, ambas causaram alterações morfofisiológicas nos animais. Entre as alterações, destacam-se a redução das hemácias, aumento dos leucócitos e da expressão das enzimas aspartato aminotransferase e alanina aminotransferase dependente da dose no soro, aumento de creatinina e de ácido úrico dependente da dose, redução da massa corporal, aumento do peso dos órgãos: fígado, rins, baço, pulmões, cérebro, coração e redução da massa dos testículos.

Quanto à hepatotoxicidade, no trabalho de Ergenekon, Erman e Şimşek Özek, (2024), realizado com cultura de células do fígado de camundongos e de humanos, foi observado que de acordo com o aumento da dose, as células hepáticas apresentavam redução na sua proliferação e do seu crescimento; entretanto a hepatotoxicidade foi maior nas culturas de células dos camundongos. Dados semelhantes foram encontrados no relatório publicado pela European Food Safety Authority (EFSA, 2011), em que a exposição ao CL parece ser menos tóxica em baixas concentrações, mas pode promover hepatotoxicidade e anemia nos modelos animais testados, de acordo com o aumento da dose ou concentração.

Além dessas informações sobre a toxicidade do CL, no trabalho de Li *et al.*, (2025), foram avaliados diversos parâmetros, como a toxicocinética, excreção e distribuição nos tecidos para os dois enatiômeros do CL o R-(-)-cletodim e o S-(+)-cletodim em roedores (ratos e camundongos). Foi demonstrado que ambos os enatiômeros apresentaram tempo de meia-vida semelhantes com média de 2 h e maior concentração localizada em órgãos do sistema digestivo (intestino e fígado). Quanto à excreção, foi observado que após 72 h restaram valores inferiores a 2% desses compostos nos tecidos. Após 96 h somente o S-(+)-cletodim foi detectado na urina, mostrando que o R-(-)-cletodim é excretado mais rápido pela urina e fezes (Li *et al*., 2025).

Como observado na literatura, os poucos trabalhos existentes já apontam para possíveis efeitos deletérios causados por esse composto, que vem sendo amplamente utilizado nas lavouras. Entretanto, seus possíveis efeitos sobre o SNC e consequências comportamentais causadas após a exposição crônica a esse composto ainda permanecem desconhecidos. Diante dessas informações, estudos que busquem preencher essas lacunas sobre seus efeitos de toxicidade e neurotoxicidade se fazem necessários, por meio de avaliações sobre as alterações comportamentais e neuroquímicas em modelos animais que busquem mimetizar a situação das pessoas expostas diretamente nas lavouras.

3. OBJETIVOS

3.1 OBJETIVO GERAL:

Avaliar as alterações comportamentais e fisiológicas após exposição repetida de cletodim em ratos.

3.2 OBJETIVOS ESPECÍFICOS:

Avaliar as alterações motoras, cognitivas e emocionais após a exposição repetida de cletodim em ratos;

Avaliar as alterações no consumo alimentar, massa corporal e massa dos órgãos após a exposição repetida de cletodim em ratos;

Avaliar as alterações glicêmicas após a exposição crônica de cletodim em ratos;

Avaliar se a exposição crônica ao cletodim altera a imunorreatividade das proteínas BDNF, c-Fos e ChAT em áreas encefálicas.

4. ARTIGO

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Repeated Clethodim Exposure Induces Behavioral Alterations and Reduces Hippocampal BDNF and c-Fos in Rats

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Abstract

Clethodim (CL) is a cyclohexanedione oxime herbicide that inhibits acetyl-CoA carboxylase (ACCase) in grasses and is widely used to control glyphosate-resistant weeds. Despite its extensive agricultural application, data regarding the toxicological effects of CL on non-target organisms remain scarce. In this sense, this study aimed to evaluate behavioral and neurochemical alterations following repeated exposure to CL in rats. Adults Wistar male rats (6-8 months old, 350 - 500g) were assigned to four groups: control (CTL - 0.9% saline solution), CL at concentrations of 0.06% (CL-0.06), 0.3% (CL-0.3), and 0.6% (CL-0.6). Animals were exposed to nebulized CL or saline every 48 h for a total of 25 sessions. Behavioral performance was evaluated using catalepsy (every 48 hours), open field (OF - days 0 and 38), novel object recognition (NOR - day 12), and spontaneous alternation (SA - day 16) tests. Metabolic parameters, organ mass, and glucose tolerance were also evaluated in addition, body mass (every 4 days), food intake (day 44), glycemic tolerance (day 48), organ mass, and brain removal (day 50) were assessed. Brains were processed for immunohistochemical analysis of BDNF, c-Fos, and choline acetyltransferase (ChAT). Repeated CL exposure induced concentration-dependent behavioral and metabolic alterations. The CL-0.06 group exhibited reduced catalepsy duration and decreased spleen mass. Animals exposed to the CL-0.6 showed anxiety-like behavior, working memory impairment, increased body mass, and a reduced glycemic peak during glucose tolerance testing. Across all CL concentrations, animals displayed reduced food intake and increased immobility behavior. Neurochemical analyses revealed decreased BDNF and c-Fos immunoreactivity in hippocampal regions in the CL-0.06 and CL-0.3 groups, whereas in ChAT expression remained unchanged. In conclusion, repeated exposure to CL induces dose-dependent behavioral, metabolic, and hippocampal neurochemical alterations, suggesting potential neurotoxic effects of this herbicide in non-target organisms.

Keywords: Pesticides, Neurotoxicity, Neurotrophins, Memory, Hippocampus.

1. Introduction

Pesticides, are known for induced toxicity in animals through the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (Čadež et al., 2021; Thapa et al., 2017). This inhibition is significantly harmful to living beings, particularly to the nervous system, which, depending on the concentration and exposure time, can overload the cholinergic system, producing increased production of free radicals, increased oxidative stress, and causing neurotoxic effects (Naughton and Terry, 2018). The accumulation can cause continuous depolarizations, as the imbalance of its levels can alter the levels of other neurotransmitters, such as GABA, glutamate, and dopamine, which can alter the neuronal excitability (Faro et al., 2022; Martínez et al., 2018; Petreski et al., 2020). Chronic exposure has been associated with the development of various autoimmune diseases, cancer, and mental health issues such as anxiety and depression. Additionally, they may contribute to neurodegenerative diseases, suicidal ideation, and endocrine system alterations (Rodrigues et al., 2025; Salcedo-Arteaga and Schuler-Faccini, 2022).

The rapid emergence of glyphosate-resistant weed species has become a major challenge for modern agriculture. Glyphosate is one of the most extensively used herbicides worldwide; nevertheless, its efficacy has declined because of the rapid development of resistance in several grass species (Heap and Duke, 2018). This scenario has driven a substantial increase in the use of alternative post-emergent herbicides, particularly clethodim (CL), which is now widely applied for the control of resistant grass species (Merotto et al., 2022; Sandín-España et al., 2012).

Clethodim ((+/-)-2-[(E)-1-[(E)3-chloroallyloxymino]propyl]-5-[2-(ethylthio) propyl]-3-hidroxy-2-cyclohexen-1-one) belongs to the cyclohexanedione oxime of herbicides group, and acts by inhibiting acetyl-CoA carboxylase specifically in monocotyledonous plants, including annual and perennial grasses, leading to impaired lipid synthesis and plant death (Rendina and Felts, 1988). Owing to its selectivity, CL is extensively used in broadleaf cropping systems and has become an important component of weed management strategies (Sandín-España et al., 2012). Despite its widespread agricultural application, information regarding the toxicological effects of CL on non-target organisms remains limited.

Available evidence suggests that CL induces adverse effects across distinct biological systems. In zebrafish, CL exposure causes head, ocular, and tail malformations, reduces AChE activity, increases oxidative stress, immunotoxicity, and mortality in dose-dependent manner (Wang et al., 2019; Xiong et al., 2019). In mice, CL has been shown to reduces testosterone synthesis in Leydig cells, decreases germ cells, and impairs sperm motility (Dcunha et al.,

2023). In rats, it alters hematological parameters, increases hepatic and renal enzyme levels, modifies body and organ mass, and reduces testicular weight (Mohamed et al., 2021). Cytotoxic and hepatotoxic effects have also been reported in murine and human liver cell models (Ergenekon et al., 2024). Though, the potential neurobehavioral and neurochemical consequences of repeated CL exposure remain poorly characterized.

Growing evidence indicates that pesticide exposure has the ability to cross the blood-brain barrier and disrupt central nervous system function by affecting neurotrophic support and neuronal activation. Brain-derived neurotrophic factor (BDNF) plays a key role in synaptic plasticity, neuronal communication, neuroprotection, and memory formation (Chapleau et al., 2009; Li et al., 2019; Mahmoud et al., 2019), with reductions in its levels been associated with cognitive and emotional impairments following toxic insults (Bekinschtein et al., 2014; Colucci-D'amato et al., 2020).

Decreased levels of this neurotrophin resulting from pesticide exposure including heightened oxidative stress, neuroinflammation, depressive-like behaviors, and memory deficits (Dorri et al., 2015; Li et al., 2019; Ribeiro et al., 2022). Likewise, the immediate early gene c-Fos is widely used as a marker of neuronal activation and is critically involved in learning- and memory-related processes (Giese, 2012; Sagar et al., 1988). Alterations in BDNF and c-Fos expression have been reported following exposure to various neurotoxic compounds, suggesting their sensitivity as indicators of functional brain alterations (Imamura et al., 2005; Kubo et al., 2022; Liu et al., 2021; Rodriguez and Giordano, 2017; Varma et al., 2024; Wu, Aiguo; Liu, 2003).

Given the increasing use of CL and the paucity of data regarding its effects on the central nervous system, this study aimed to evaluate the behavioral, physiological, and neurochemical consequences of its repeated exposure in rats, with particular emphasis on changes in BDNF, c-Fos, and choline acetyltransferase (ChAT) expression.

2. **Material and methods**

2.1 Animals

Thirty male adult Wistar rats (6-8 month-old; 350-500 g) were utilized. The rats were obtained from the animal facility of the Department of Physiology at the Federal University of Sergipe (UFS) and were housed in the animal facility of the Laboratory of Behavioral and Evolutionary Neurobiology at UFS. The animals were randomly housed in four groups in standard polypropylene cages (33 x 40 x 17 cm) (four animals per cage), under controlled conditions of acoustic insulation, airflow and temperature (22 ± 2ºC), humidity and luminosity

(12 h light/12 h dark cycle, lights on 06:00 a.m.) with free access to water and food. All care and procedures for the animals in this study adhered to the principles established by Brazilian law for the use of animals in research, specifically Law No. 11.974/08. All procedures were conducted following approval from the Ethics Committee on the Use of Animals at UFS (CEUA/UFS) under protocol number 5228100323. All efforts were made to minimize the number of animals used and to reduce pain, suffering, and discomfort.

2.2 Chemicals

A commercial formulation of clethodim (Select® 240 EC, Paraná, Brazil) was used. The concentrations chosen for this study began with the manufacturer's recommended concentration, which is commonly used by farmers (0.06%). This was followed by two higher concentrations: one five-fold the initial concentration (0.3%) and another that is ten-fold more concentrated (0.6%). All concentrations were diluted in 0.9% saline solution and stored in 200 mL amber bottles for further use.

2.3 Experimental design

The animals participated in daily handling sessions lasting 5 minutes each for 5 consecutive days before the start of the experiment. This was done to help them acclimate to being touched and to the presence of the experimenters. All behavioral tests were conducted during the light phase (08:00 a.m. – 1:00 p.m). Before each behavioral session, animals were habituated to the testing room for 30 min. Behavioral apparatuses were cleaned with a 10% alcohol solution between sessions, and experiments were video-recorded with a digital camera (Logitech C920e Full Hd 1080p, Lausanne, Switzerland) and analyzed using ANY-maze software (v7.51, Stoelting Co., Wood Dale, IL, USA).

Animals were randomly assigned to four experimental groups: (1) Control (CTL, 0.9% saline; n = 7); (2) CL-0.06 (0.06% clethodim; n = 7); (3) CL-0.3 (0.3% clethodim; n = 8), and (4) CL-0.6 group (0.6% clethodim; n = 8). To model environmental exposure, CL or saline was administered via nebulization using an ultrasonic nebulizer (C801NEBLA, OMRON Healthcare, Inc., Hoffman Estates, IL, USA) placed over a sealed exposure chamber (33 × 40 × 17 cm). Nebulization sessions lasted 10 min and were performed every 48 h for a total of 25 sessions. A volume of 4 mL of solution was used per session, and with this volume, no wet fur was observed in the animals. Each housing cage contained only animals from the same experimental group and concentration.

The nebulization sessions were consistently scheduled following the behavioral tests, allowing us to evaluate the effects of the prior nebulization. Throughout the process, the nebulization box was maintained within the fume hood to prevent any airborne dissemination of the compounds used. We employed two nebulization boxes of identical size and shape, ensuring that the control animals were always housed two at a time in a box that never came into contact with the experimental group.

The commercial formulation was diluted only twice over the 50 days and was stored in amber glass containers wrapped in aluminum foil to protect it from photodegradation. The order of nebulization for the animals was as follows: first, all control (CTL) individuals, followed by the CL-0.06, CL-0.3, and CL-0.6 groups, respectively. After the 10 minutes of nebulization, the box was left open in the fume hood for an additional two minutes to allow any remaining CL to dissipate. The animals were then placed in individually identified boxes lined with wood shavings to eliminate any residual smell of saline solution or CL for 30 minutes. After this period, the animals were returned to their designated cages and taken to the animal facility.

After each animal left the nebulization box, all materials were thoroughly sanitized. During the nebulization process, the animals were closely monitored inside the nebulization box for the entire scheduled duration. This was to ensure that if they displayed signs of hyperactivity, emitted sounds indicating discomfort, showed drowsiness, or struggled inside the box, they could be promptly removed from the experiment. Fortunately, none of the animals exhibited these behaviors.

Throughout the experiment, the animals were subjected to several tests, assessments, and procedures: (1) catalepsy (1 h before the 1^{st} – on day 0 - and 48 h after each nebulization); (2) open field (1 h after the 1^{st} - on day 0 - and 48 h after the 19^{th} nebulization - on day 38); (3) forced swimming (24 h after the 13^{th} nebulization - on day 25); (4) body mass assessment (on day 0 and then every 4 days); (5) novel object recognition (48 h after the 5^{th} nebulization - on day 12); (6) spontaneous alternation (48 h after the 8^{th} nebulization - on day 16); (7) food intake assessment (48 h after the 19^{th} nebulization - on day 38); (8) glucose tolerance (48 h after the 24^{th} nebulization - on day 48); (9) perfusion (48 h after the 25^{th} nebulization - on day 50); (10) assessment of the mass of the perfused liver, lungs, spleen and testicles (48 h after the 25^{th} nebulization - on day 50, after the perfusion) **(Fig. 1)**. All acquisition and analyses were performed by an investigator blinded to the experimental groups.

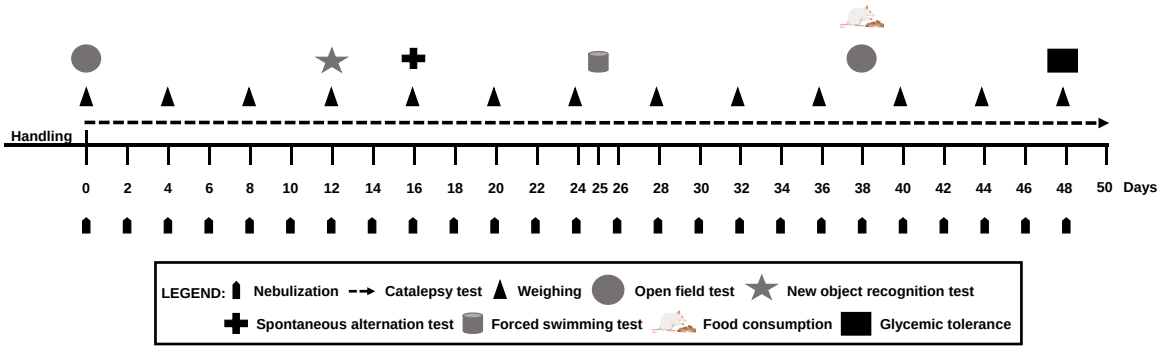

Figure 1: Schematic representation of the experimental design. The experiment was conducted over a period of 50 days, during which the animals received 25 sessions of nebulization with saline solution or CL. The tests and procedures performed are shown in the figure.

2.4 Behavioral Assays

2.4.1 Catalepsy Test

Catalepsy test was assessed as a measure of motor impairment. Animals were positioned with both forepaws placed on a horizontal metal bar elevated 9 cm above the surface. The latency, to initiate movement and remove at least one paw from the bar was recorded. Each animal was subjected to three consecutive trials per session, with a maximum duration of 180 s per trial. Catalepsy assessments were performed every 48 h, and the mean latency across the three trials was used for analysis, as previously described (Melo et al., 2022; Santos et al., 2013).

2.4.2 Open field

The open field (OF) testwas used to evaluate locomotor activity and behavioral anxiety-like responses for 5 min (Walsh and Cummins, 1976). Animals were individually placed in the center of a circular arena (80 cm radius, 60 cm high) and their behavior was video-recorded for 5 min for subsequent analysis. It has no roof, and the interior is painted matte black to enhance visibility during test recordings. A camera was mounted above the apparatus at a height of 240 cm, connected to a computer, allowing for the recording of behavioral parameters. All animals are placed in the center of the apparatus, and the parameters evaluated include the time spent in the center and the total distance traveled, as previously described (Melo et al., 2022; Souza et al., 2022).

2.4.3 Spontaneous alternation

Spontaneous alternation was used to assess working memory. The test was conducted in a four-arm maze with four-arm maze with laterally enclosed arms (48 cm long, 14 cm wide, and 28 cm high). Animals were placed in the central area of the apparatus, and allowed to freely

explore the arms freely for 5 min. The sequence of arm entries was recorded, and a correct alternation was defined as consecutive entries into all four arms without repetition. Working memory performance was expressed as the percentage of spontaneous alternation, as previously described (Souza et al., 2020).

2.4.4 Novel object recogniton

The novel object recognition test (NOR) was used to assess short-term recognition memory (Ennaceur and Delacour, 1988; adapted from (Souza et al., 2018). The task was conducted in the open field apparatus. Animals were habituated to the arena for 5 min on the day preceding the test. During the training phase, animals were allowed to freely explore two identical objects for 5 min (training). After a 1 h retention interval, animals were re-exposed to the arena containing one familiar object and one novel object for an additional 5 min (test). The exploration time with the objects was considered as the time spent by the animals interacting with the objects, such as touching them with their front paws, nose, smelling, or biting the objects. Based on this, the exploration time was quantified (for the old or new object/exploration time for both objects) to assess short-term memory.

2.4.5 Forced swin test

The forced swim test was used to assess depression-like and stress-coping behaviors, as well as for preclinical analysis of antidepressant activity involving drugs and animals exposed to environmental contaminants, as previously described (Petit-Demouliere et al., 2005; Porsolt et al., 1979; Santos-Carrasco et al., 2025). Animals were individually placed in a black polyethylene cylindrical tank (35 cm diameter, 40 cm height) filled with water maintained at 25 ± 1 °C for a single 5 min session. Behavioral parameters recorded included latency to the first immobility episode, total immobility time, and number of immobility events, as commonly applied in studies involving environmental exposure and neurobehavioral assessment.

2.4.6 Body mass evaluation

The weight Body mass was measured every four days using a DICOBEM portable scale. Body mass variation (%) was calculated as a percentage using the following formula M = [(Mf/Mi) -1) x 100] was used, where M represents body mass, Mf represents final body mass, and Mi represents initial body mass.

2.4.7 Organs mass

At the end of the experiment, the animals were perfused, and the spleen, liver, testicles and lungs were collected. Organs were weighed using an analytical balance. Relative organ mass was calculated by normalizing organ weight to the total body mass of each animal, using the following ratio: relative organ mass: organ mass/animal mass.

2.4.8 Food ingestion evaluation

Food intake was assessed on day 38 of the experimental protocol. The amount of food provided to each cage was weighed before and 24 h after exposure. Individual food intake was estimated by normalizing total cage consumption to the body mass of each animal, using the following formula: individual intake = (individual animal mass / total box mass) x (intake) / individual animal mass.

2.4.9 Glucose tolerance test

Glucose tolerance was assessed following a 12 h fasting period, with free access to water. Baseline blood glucose levels were measured from the tail vein using a handheld glucometer. Subsequently, animals received an oral glucose load (50% glucose solution, 1 mL/kg) via gavage. Blood glucose levels were measured at 30, 60, and 120 min post-gavage, as previously described (Boaventura et al., 2023).

2.4.10 Perfusion

At the conclusion of the behavioral tests on day 50, the animals were anesthetized with intraperitoneal injections of ketamine and xylazine hydrochloride at dosages of 100 mg/kg^{-1} and 10 mg/kg^{-1}, respectively. Reflex assessments were conducted to ensure the animals were fully anesthetized. After confirmation of deep anesthesia, animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4) for 10 min, followed by 4% paraformaldehyde in PB, pH 7.4 for 20 min to fix the tissue. Following perfusion, the liver, spleen, lungs, and testicles were removed. The brains were extracted from the skull and post-fixed in a 4% paraformaldehyde and sucrose 30% solution for 24 h at 4°C, and subsequently cryoprotected in 30% sucrose until saturation. Brain tissue were then frozen at -20°C and sectioned into 50 µm coronal sections using a cryostat (CM1850, Leica Biosystems Inc., Buffalo Grove, IL, USA). The distance between consecutive sections within the same compartment was

approximately 200 μm. All sections were stored in an antifreeze solution (30% ethylene glycol and 20% glycerol in 0.2 M PB, pH 7.4) at -20°C until further processing (de Gois et al., 2025).

2.4.11 c-fos, BDNF and ChAT Immunohistochemistry

Immunohistochemistry was performed at room temperature (22–25°C) using free-floating sections. Tissue sections were rinsed in 0.1 M phosphate buffer (PB; 4x, 5 min each) and incubated in 0.03% hydrogen peroxide (H_2O_2) in 0.1 M PB to block endogenous peroxidase activity, followed by blocking in 5% skim milk in 0.1 M PB solution to reduce nonspecific binding. Sections were then incubated overnight (18 h) with the following primary antibodies diluted in PB containing 0.4% Triton X-100: anti-c-Fos (monoclonal IgG, Santa Cruz Biotechnology, SC-166940, Lot #D2318, produced in mouse, 1:500), anti-BDNF (cat #AB152 Santa Cruz, USA, 1:600) produced in sheep, and anti-ChAT produced in goat (cat. no. AB144P Chemicon, USA, 1:1000). After washing, sections were incubated with the appropriate biotinylated secondary antibodies diluted in PB containing 0.4% Triton X-100 for 2 h: anti-mouse produced in rabbit (1:500; Sigma Chemical Company), anti-sheep produced in donkey (1:1000; Sigma Chemical Company) and anti-goat produced in rabbit (1:750; Sigma Chemical Company). The sections were washed with PB and then incubated with the avidin-biotin enzyme complex at dilutions of 4:1000 (ABC Elite kit from Vector Labs, Burlingame, USA) for 2 hours. Following this, the sections were washed visualized using a solution of 2.5% diaminobenzidine tetrachloride (DAB, Sigma, USA) diluted in 0.1 M PB (pH 7.4) along with 0.03% hydrogen peroxide. Reaction times were optimized for each antibody. Sections were mounted on gelatin-coated slides, intensified with osmium tetroxide (0.005%), dehydrated through graded alcohols, cleared in xylol, and coverslipped with Entellan (Merck, Darmstadt, Germany).

2.4.12 Microscopy and image analysis

Immunolabeled sections were examined under bright-field microscopy using a Nikon Eclipse Ci-S microscope equipped with a digital camera and NIS-Elements software (Nikon Corporation Inc., Tokyo, Japan). Anatomical regions of interest were identified according to the rat brain atlas of Paxinos and Watson (2007). For c-Fos analysis, images were acquired at 100× magnification from four non-adjacent coronal sections per animal in the following regions: medial prefrontal cortex (mPFC), insular cortex (IC), CA1, CA3, and dentate gyrus (DG). Quantification of c-Fos–positive cells was performed using NIH ImageJ software (https://imagej.net/ij/). Cell counts were averaged per animal and normalized to the mean values

of the control group. For BDNF (mPFC, IC, medial septum, CA1, CA3, and DG) and ChAT (medial septum and nucleus basalis of Meynert) immunoreactivity, optical densitometry (OD) analysis was conducted. Four images of each section were acquired under identical illumination conditions, and mean optical density values were obtained using ImageJ. Six animals per group were used for the analyses, but some areas could not be photographed, resulting in the use of 4 to 6 animals per treatment. Data were normalized to control group values, as previously described (Santos et al., 2013). All image acquisition and analyses were performed by an investigator blinded to the experimental groups.

2.4.13 Statistical analysis

Data were assessed for normality using the Shapiro-Wilk test. As assumptions for parametric analyses were met, appropriate parametric tests were applied. Two-way ANOVA with repeated measures, followed by Fisher's LSD *post-hoc* test, was used for catalepsy performance, body mass variation, and glycemic tolerance data. One-way ANOVA was applied to analyze open field, spontaneous alternation, food intake, and organ mass. Novel object recognition (NOR) test was analyzed using a paired t-test. Pearson's correlation analysis was carried out to examine associations between food intake and body mass, as well as between BDNF immunoreactivity and c-Fos expression. Data are presented as mean ± standard error of the mean (± SEM), with significance set at $p < 0.05$. All analyses were conducted using GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, CA, USA).

3. **Results**

3.1 Behavioral Assays

3.1.1 Catalepsy test

In the catalepsy test, the two-way ANOVA revealed significant effects of both time (days of treatment [$F (25, 676) = 14.37$; $p < 0.0001$] and treatment (CTL, CL-0.06, CL-0.3 and CL-0.6) [$F (3, 676) = 7.579$; $p < 0.0001$], but not interaction effect (time x treatment) [$F (75, 676) = 0.5275$; $p = 0.9996$]. Fisher LSD post-test showed significant differences between the groups at three time points. On day 32, the CL-0.6 group spent more time on the bar compared to the CL-0.06 group ($p = 0.0463$). On day 34, the CL-0.06 group spent less time on the bar than the CTL group ($p = 0.0191$). On day 50, the CL-0.06 group again spent less time on the bar compared to both the CL-0.6 ($p = 0.0039$) and CTL ($p = 0.0279$) groups (**Fig. 2A**). In the

area under the curve assessment, the one-way ANOVA did not indicated significant treatment effect between the groups [F (3, 26) = 1.012; p = 0.4032] (p < 0.0001) (**Fig. 2A'**).

3.1.2 Open field test

In the open field test, conducted on day 0, one-way ANOVA reported a treatment effect (F (3,26) = 4.243; p = 0.0144) for the time in center parameter. Using Fisher's LSD *post-hoc* test, the CL-0.6 group showed less time in center than the other experimental groups: CTL (p = 0.0027); CL-0.3 (p = 0.0131); CL-0.06 (p = 0.0277). Regarding the total distance traveled parameter, one-way ANOVA did not report a treatment effect between groups (F (3,26) = 1.649, p = 0.2024). In the open field test on day 38, one-way ANOVA did not show a treatment effect between groups for time in center [F (3, 26) = 2.368; p = 0.0938] and for the total distance traveled [F (3, 26) = 0.4773; p = 0.7008] (**Table 1**).

Table 1: Effects of Acute and Chronic Exposure to CL (0.06%, 0.03%, and 0.6%) on Anxiety-Like Behavior and Motor Activity in Adult Rats in the Open Field Test.

Parameters	Day	CTL	0.06	0.3	0.6
Total distance traveled (m)	0	16.41 ± 0.96	11.36 ± 2.39	13.80 ± 1.49	13.68 ± 1.07
Time at the center (s)	0	2.23 ± 0.27	1.79 ± 0.39	1.94 ± 0.39	1.14 ± 0.18***ab**
Total distance traveled (m)	38	4.98 ± 1.02	6.91 ± 1.22	6.14 ± 1.29	6.76 ± 1.32
Time at the center (s)	38	3.07 ± 0.55	7.92 ± 2.92	6.86 ± 2.41	13.24 ± 3.69

Values are expressed as mean ± standard error of the mean (SEM). *p < 0.05 compared to the control (CTL) group; ap < 0.05 compared to the CL-0.06 group; bp < 0.05 compared to the CL-0.03 group, determined by one-way ANOVA followed by Fisher's LSD *post-hoc* test.

3.1.3 Novel object recognition test

In the NOR test, paired-samples t-test indicated that exposure to CL did not lead to short-term memory deficits. All groups exhibited significantly longer exploration times for the novel object compared to the familiar object. The results were as follows: CTL [t (6) = 2.618; p = 0.0397]; CL-0.06 [t (6) = 3.516; p = 0.0126]; CL-0.3 [t (7) = 3.004; p = 0.0198]; and CL-0.6 [t (8) = 2.365; p = 0.05] (**Fig. 2B**).

3.1.4 Spontaneus alternation test

On day 16 of the SA test, which examined the effects of the eighth exposure, one-way ANOVA revealed a significant treatment effect between the groups [F (3, 26) = 4.442; p = 0.0120]. Fisher's LSD *post-hoc* test showed that animals of CL-0.6 group had a reduction in the number of correct alternations when compared with CTL (p = 0.0112) and CL-0.06 (p = 0.0027) groups (**Fig. 2C**).

3.1.5 Forced swim test

In the forced swim test, one-way ANOVA did not reveal a significant treatment effect [F (3, 26) = 1.969; p = 0.1434] for latency to the first stop. However, when examining the number of immobility events, a significant treatment effect was observed [F (3, 26) = 3.268; p = 0.0372]. All CL-treated groups exhibited a greater number of immobility events than the CTL group, with significant increases for CL-0.6 (p = 0.0097), CL-0.3 (p = 0.0417), and CL-0.06 (p = 0.0145) (**Fig. 2D'**). A significant treatment effect was found for total immobility time [F (3, 26) = 3.600; p = 0.0268], with all CL-treated groups spending more time immobile than the CTL group. Significant increases were observed for CL-0.6 (p = 0.0165), CL-0.3 (p = 0.0254), and CL-0.06 (p = 0.0056) (**Fig. 2D''**).

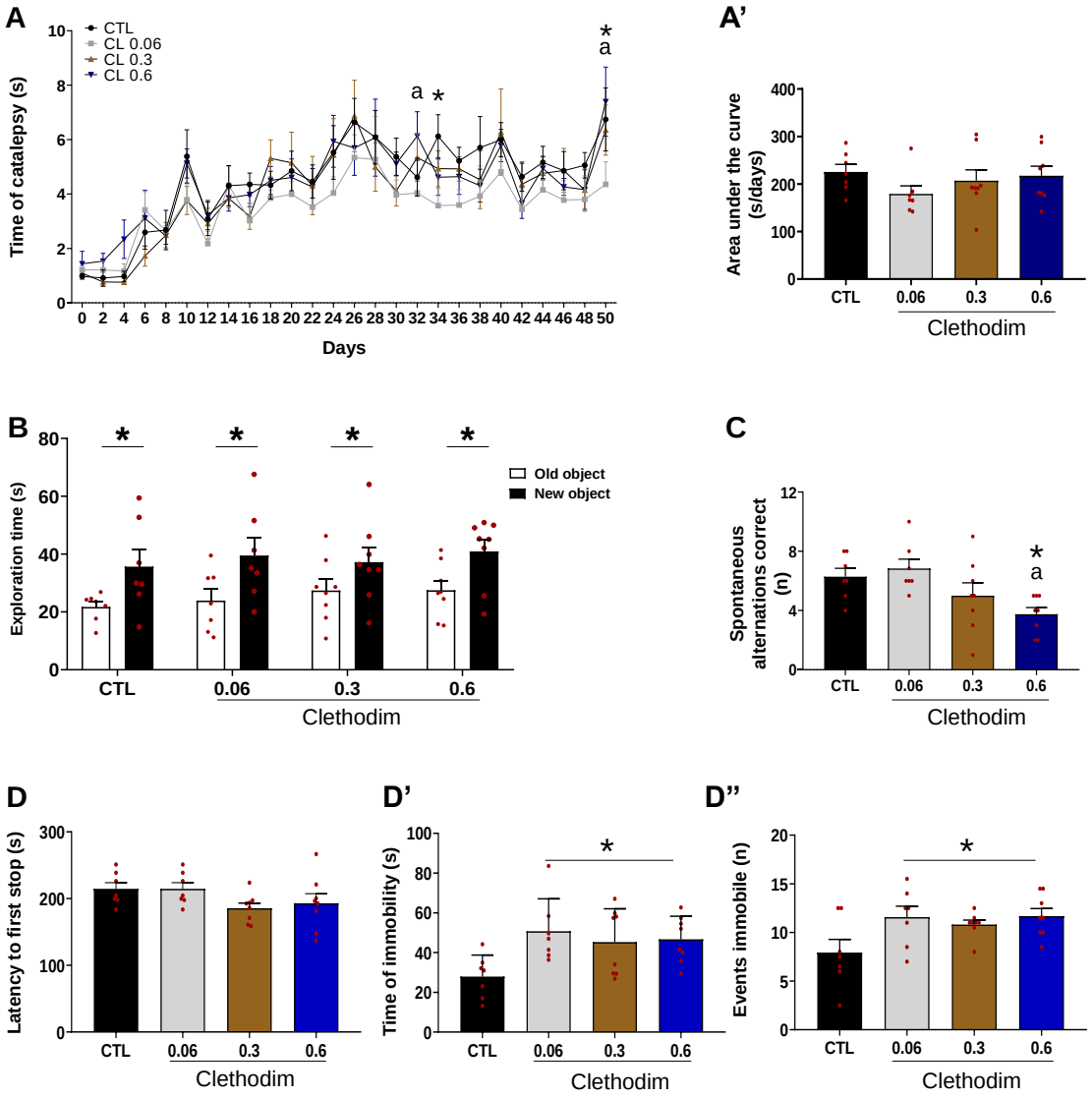

Figure 1: Effect of chronic exposure to CL (0.06%, 0.03%, and 0.6%) in adult rats on behavioral tests of catalepsy, new object recognition, spontaneous alternation and forced swimming test. A – Time on the bar in the catalepsy test, A' area under the curve. B – Exploration time in the RO test. C – Number of correct changes made in the SA test. D – Latency to first stop in FST, D' – Time of events immobile, D'' – number of immobile events. Values are expressed as mean ± standard error of the mean (SEM) ∗p < 0.05 compared to the CTL group; ap < 0.05 compared to the CL-0.06 vs CL-0.6 group; bp < 0.05 compared to the CL-0.06 vs CL-0.3 group, by one-way ANOVA followed by Fischer LSD *post-hoc* test.

3.2 Metabolic parameters

3.2.1 Glucose tolerance test

In the examination of glycemic tolerance, two-way ANOVA with repeated measures revealed significant effects: time [F (3, 104) = 38.09; p = 0.0001], treatment [F (3, 104) = 11.88; p = 0.0001] and interaction effects [F (9, 104) = 2.991; p = 0.0033]. Thirty minutes after glucose administration, all groups, except the CL-0.6 group (p = 0.0594), exhibited an increase in blood glucose levels compared to the baseline (time 0). At this 30-minute mark, both the CL-0.06 (p = 0.017) and CL-0.6 groups (p = 0.0008) showed a lower glucose level compared to the CTL

group. The CL-0.3 group demonstrated increased glucose levels compared to the CL-0.06 group ($p = 0.0124$). In contrast, the CL-0.6 group showed reduced glucose availability compared with both the CL-0.06 ($p = 0.0024$) and CL-0.3 groups ($p = 0.0006$). Notably, only at the 60 minutes time point the CL-0.6 group showed an increase in glucose availability compared to baseline ($p = 0.0007$). For the other groups, no significant differences were observed between 60 to 120 minutes. By the end of the test, all groups returned to their baseline values (**Fig. 3A**).

In addition, we performed an analysis of the area under the curve, and the one-way ANOVA reported a treatment effect between groups [$F (3, 36) = 11.55$; $p = 0.0001$], demonstrating that the CL-0.06 and CL-0.6 groups had a smaller area when compared to the CTL ($p = 0.0032$, $p = 0.0002$; respectively) and CL-0.3 ($p = 0.0012$, $p < 0.0001$; respectively) (**Fig. 3A'**).

3.2.2 Food ingestion

In the assessment of food intake, one-way ANOVA reported treatment effect [$F (3, 26) = 23.64$; $p < 0.0001$]. According to Fisher's LSD post-test, all CL groups exhibited reduced compared with the CTL group (CL-0.6, $p < 0.0001$; CL-0.3, $p < 0.0001$; CL-0.06, $p < 0.0001$) (**Fig. 3B**). Furthermore, a Pearson correlation was performed between food intake and body mass gain content of the animals ($r = 0.04206$, $p = 0.8254$), revealing a non-significant relationship between the variables (**Fig. 3B'**).

3.2.3 Body mass variation

In the assessment of body mass, two-way ANOVA with repeated measures reported a time [$F (12,338) = 5.721$; $p < 0.0001$] and treatment effects [$F (3,338) = 56.58$; $p < 0.0001$], but not interaction effect [$F (36, 338) = 0.7887$; $p = 0.8047$]. From day 4 until the end of the experiment, the CL-0.6 group showed the greatest gain in total body mass when compared to the other groups (**Fig. 3C**).

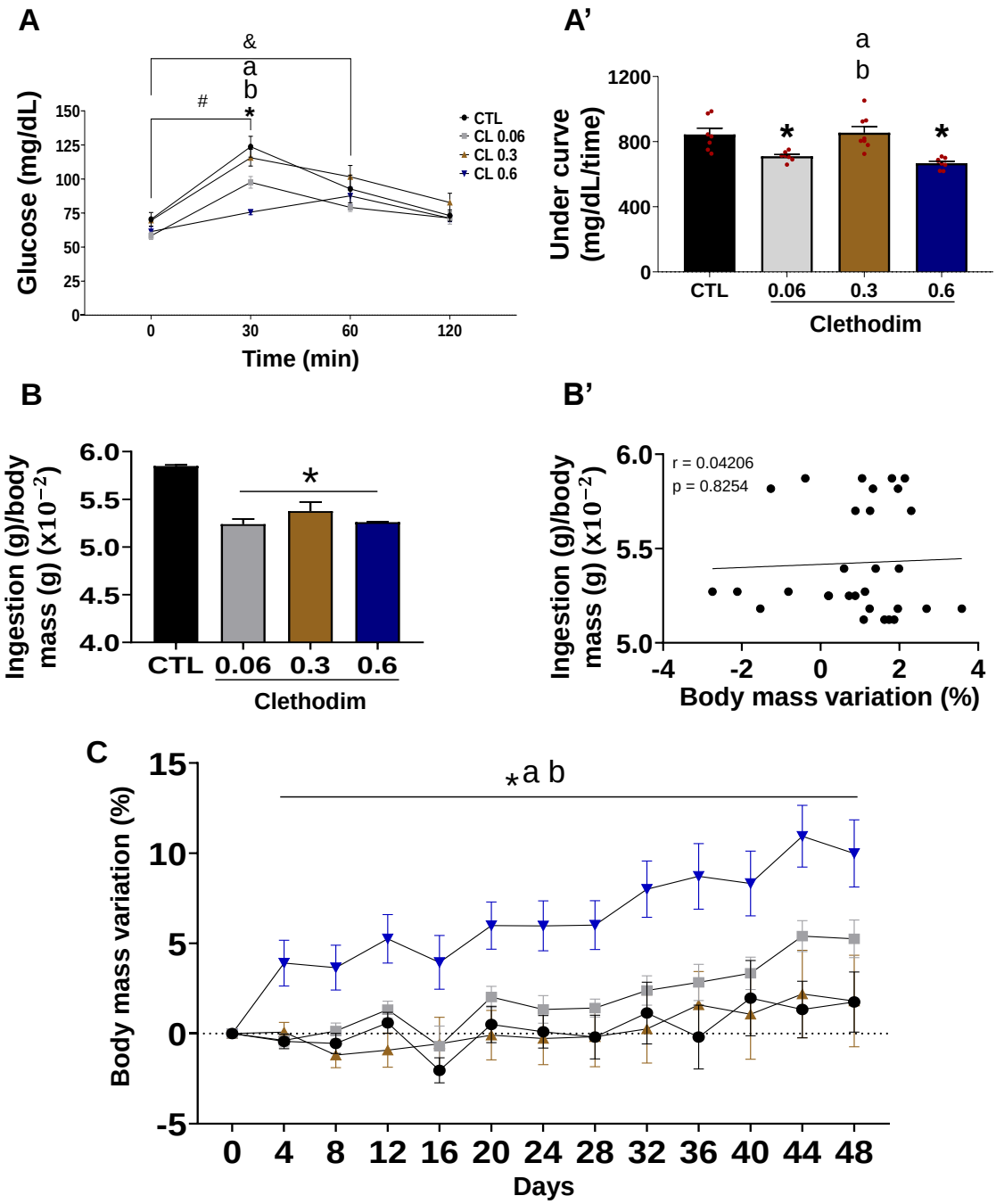

Figure 2: Effect of chronic exposure to CL (0.06%, 0.03%, and 0.6%) on metabolic parameters, including (A) glycemic tolerance, (A') Under curve the glycemic tolerance, (B) food intake, (B´) food intake vs. body mass and (C) body mass. Values are expressed as mean ± standard error of the mean (SEM). In tolerance the glucose: ∗p < 0.05 compared to the CTL group; ap < 0.05 compared to the CL-0.06 time 30 vs time 0; bp < 0.05 compared to the CL-0.3 time 30 vs time 0; #p > 0.05 compared to the CL-0.6 in 0 time vs 30 time; &p < 0.05 compared to the CL-0.6 in time 60 vs 0 time. Ingestion / mass and body mass variation: ∗p < 0.05 compared to the CTL group; ap < 0.05 compared to the CL-0.6 vs CL-0.06 group; bp < 0.05 compared to the CL-0.6 vs CL-0.3 group, by one-way ANOVA followed by Fischer LSD *post-hoc* test.

3.2.4 Organ mass

In the evaluation of the organs mass, one-way ANOVA reported a treatment effect only on spleen [F (3, 26) = 8.212; p = 0.0005]. Fisher's LSD post-test showed that the CL-0.06 group presents lower mass when compared to the CL-0.6 (p = 0.0204), CL-0.3 (p = 0.0002) and CTL

(p = 0.0003) groups. In the evaluation of the other organs, one-way ANOVA did not show a treatment effect: liver [F (3, 26) = 0.7886; p = 0.5112]; testes [F (3, 26) = 1.081; p = 0.3746] and lungs [F (3, 26) = 0.2750; p = 0.8429] (**Table 2**).

Table 2: Effect of acute and chronic exposure to CL (0.06%, 0.03% and 0.6%) in adult rats on organ mass corrected for animal mass.

Organ	CTL	CL-0.06	CL-0.3	CL-0.6
Liver (g/g)	0.033 ± 0.002	0.032 ± 0.002	0.036 ± 0.002	0.031 ± 0.001
Spleen (g/g)	0.002 ± 0.001	0.001 ± 0.001*ab	0.002 ± 0.001	0.002 ± 0.001
Testicles (g/g)	0.008 ± 0.001	0.007 ± 0.001	0.007 ± 0.002	0.007 ± 0.001
Lungs (g/g)	0.013 ± 0.002	0.011 ± 0.001	0.017 ± 0.001	0.013 ± 0.002

Values are expressed as mean ± standard error of the mean (SEM) * p < 0.05 compared to the CTL group; ap < 0.05 compared to the CL-0.6 group; bp < 0.05 compared to the CL-0.3 group, by one-way ANOVA followed by Fisher's LSD *post-hoc* test.

3.3 Immunohistochemistry

3.3.1 c-Fos

Immunoreactivity analysis for c-Fos was performed for five areas: three hippocampal areas (CA1, CA3, and GD) and two frontal areas (mPFC and IC). One-way ANOVA did not report a treatment effect between groups for the GD region [F (3, 12) = 0.6536; p = 0.5959] (**Fig. 4A**). For the CA1 area, one-way ANOVA showed a treatment effect between groups [F (3, 12) = 5.249; p = 0.0152]. By Fisher's LSD *post-hoc* test, the CL-0.03 and CL-0.06 groups showed lower activation than the animals in the CTL group (p = 0.0019; p = 0.0442; respectively) (**Fig. 4B**). For the CA3 area, one-way ANOVA showed no treatment effect between groups [F (3, 12) = 0.2455; p = 0.8630] (**Fig. 4C**).

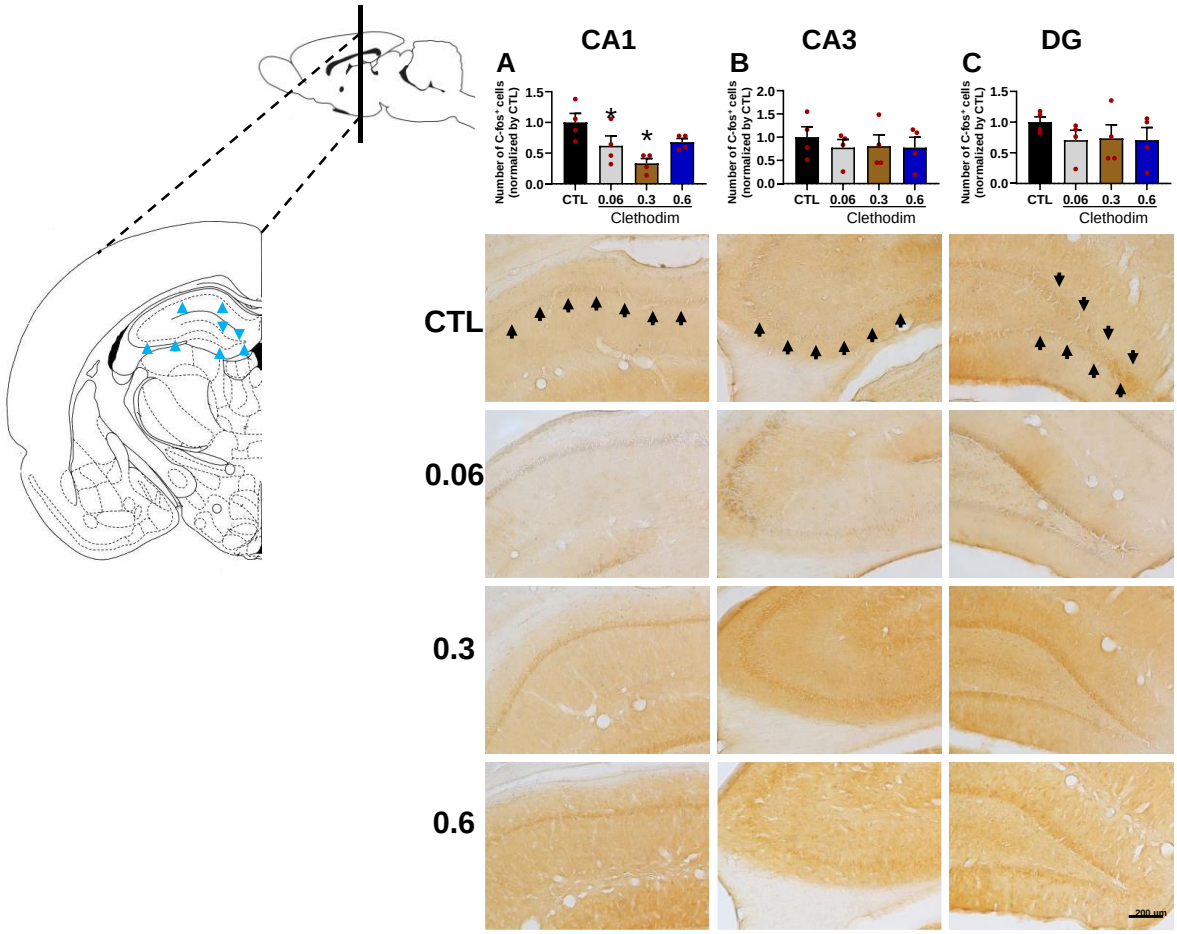

Figure 4: Effect of chronic CL exposure (0.06%, 0.03%, and 0.6%) in adult rats on immunoreactivity to c-Fos protein in hippocampal and frontal areas. A – c-Fos immunoreactivity in the dentate gyrus (DG) hippocampal region, B – c-Fos immunoreactivity in the CA1, C – c-Fos immunoreactivity in the CA3. Values are expressed as mean ± standard error of the mean (SEM) * $p < 0.05$ compared to the CTL group, by one-way ANOVA followed by Fisher's LSD *post-hoc* test.

For the frontal areas, one-way ANOVA reported no treatment effect for mPFC [F (3, 12) = 0.3296; p = 0.8041], however, it was reported for the CI area [F (3, 12) = 14.75; p = 0.0002]. By the Fisher LSD *post-hoc* test, the CL-0.6 and CL-0.06 groups showed less activation when compared to the CTL group (p = 0.0097; p = 0.0046; respectively) (**Fig. 5A**). Regarding the CL-0.3 group compared to the CL-0.6, CL-0.3 and CTL groups, an increase in its immunoreactivity was demonstrated (p = 0.0001; p = 0.0002; p= 0.0417; respectively) (**Fig. 5B**).

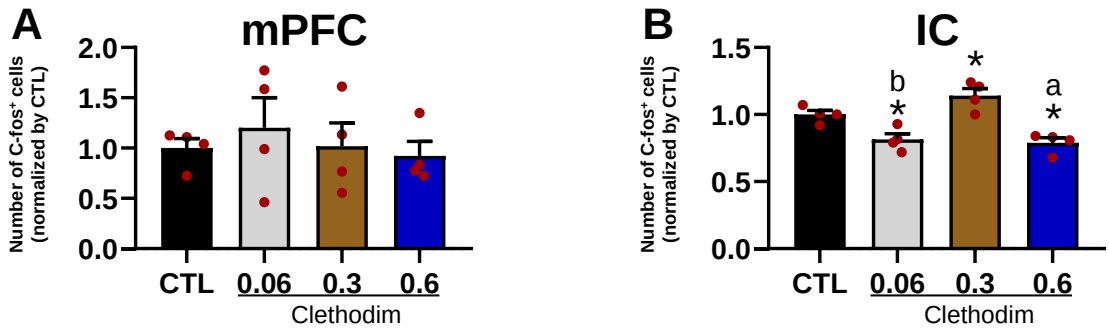

Figure 5: Effect of chronic CL exposure (0.06%, 0.03%, and 0.6%) in adult rats on immunoreactivity to c-Fos protein in frontal areas. A – medial prefrontal region, B – insular cortex region. Values are expressed as mean ± standard error of the mean (SEM) * $p < 0.05$ compared to the CTL group; bp < 0.05 compared to the CL-0.06 vs CL-0.3 group; bp < 0.05 compared the CL-0.6 group vs CL-0.3, by one-way ANOVA followed by Fisher's LSD *post-hoc* test.

3.3.2 BDNF

One-way ANOVA indicated no significant treatment effect [F (3, 16) = 0.5658; p = 0.6454] for the mPFC area (**Fig. 6A**). However, in the SEm area, treatment effect was observed [F (3, 17) = 3.303; p = 0.0456] (**Fig. 6B**). Fisher's LSD test revealed a significant reduction in BDNF levels in the CL-0.6 group compared to both the CL-0.3 and CL-0.06 groups (p = 0.0137; p = 0.0185, respectively). In the hippocampal areas, one-way ANOVA demonstrated a treatment effect in the CA1 [F (3, 15) = 3.566; p = 0.0397] (**Fig. 6C**). The Fisher's LSD post-test showed a significant decrease in BDNF protein immunoreactivity in the CL-0.3 and CL-0.06 groups compared to the control (CTL) group (p = 0.0076; p = 0.0307, respectively). In contrast, no treatment effect was found in the CA3 [F (3, 16) = 0.8558; p = 0.4839] (**Fig. 6D**) and DG [F (3, 16) = 0.7206; p = 0.5542] (**Fig. 6E**). Additionally, correlation analysis between BDNF levels and the activation of c-Fos-positive neurons revealed a positive Pearson correlation in the CA1 area (r = 0.5265; p = 0.0362) (**Fig. 6F**), indicating that reduced BDNF immunoreactivity was associated with a lower number of c-Fos-active neurons.

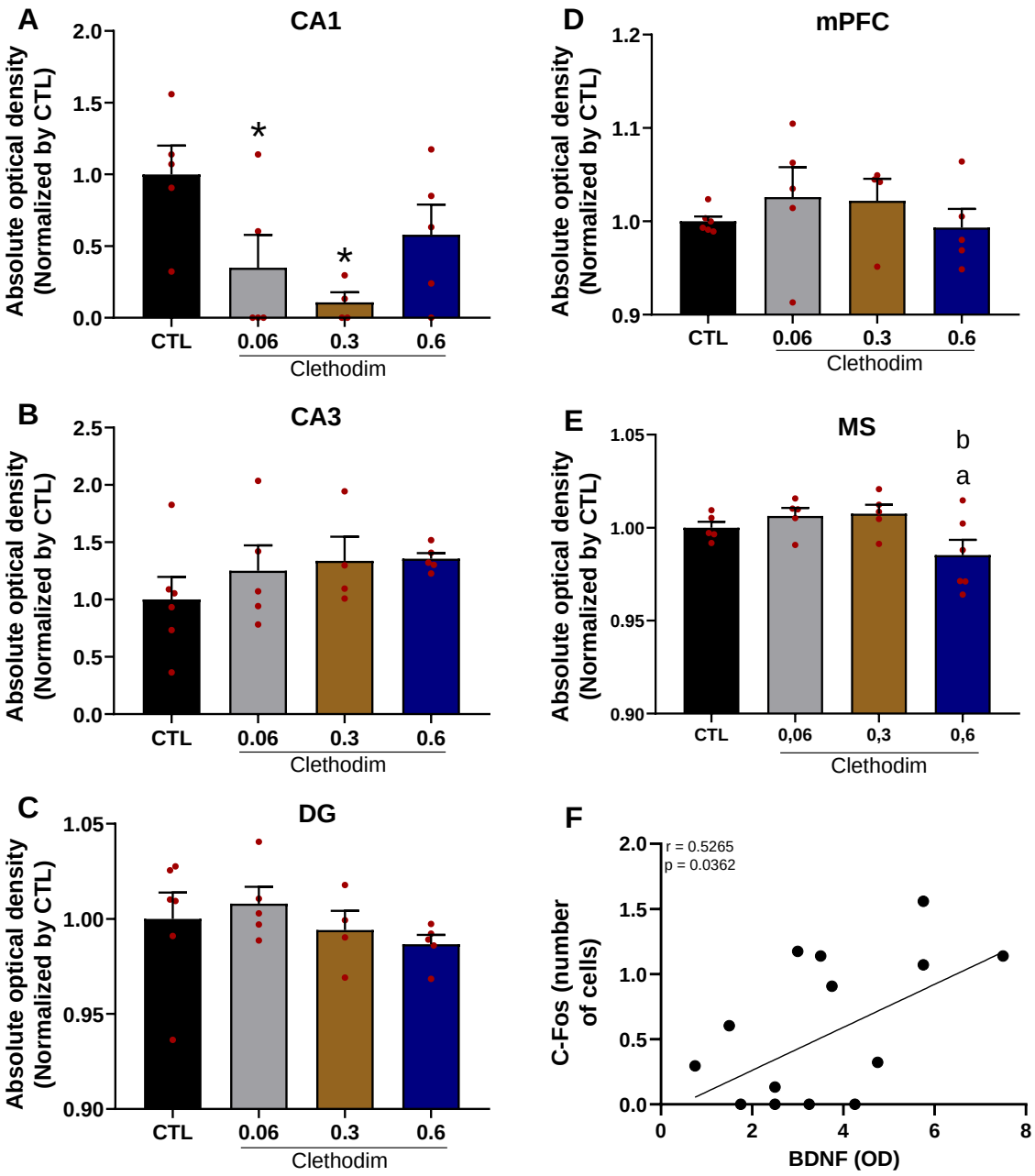

Figure 6: Effect of chronic exposure to CL (0.06%, 0.03%, and 0.6%) in adult rats on immunoreactivity for BDNF protein in frontal and hippocampal areas. A – Immunoreactivity for BDNF in the hippocampal region of Ammon's horn 1 (CA1), B – Immunoreactivity for BDNF in the hippocampal region of Ammon's horn 1 (CA1), C – Immunoreactivity for BDNF in the hippocampal region of the dentate gyrus (DG). D – Immunoreactivity for BDNF in the medial prefrontal cortex (mPFC) region, E – Immunoreactivity for BDNF in the medial septal (MS) cholinergic region, F – Correlation of BDNF vs. c-Fos levels in the CA1 area. Values are expressed as mean ± standard error of the mean (SEM) ∗p < 0.05 compared to the CTL group; p < 0.05 compared to the CL-0.06 vs CL-0.3 group; bp < 0.05 compared to the CL-0.6 vs CL-0.3 group, by one-way ANOVA followed by Fischer LSD post-test.

3.3.3 ChAT

In an immunohistochemistry examining ChAT in the cholinergic nuclei of the medial septum and the basal nucleus of Meynert, one-way ANOVA indicated no significant treatment effect for both areas: medial septum [F (3, 16) = 0.07368; p = 0.9733] (**Fig. 6A**) and basal

nucleus of Meynert [F (3, 16) = 0.3511; p = 0.7889] (**Fig. 6B**). These findings demonstrate that exposure to CL did not alter the density of ChAT in these cholinergic brain regions.

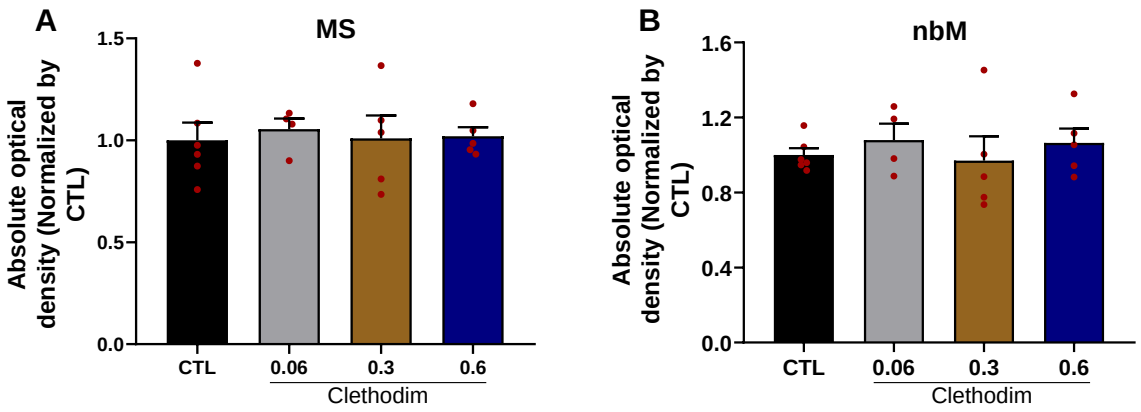

Figure 7: Effect of chronic exposure to CL (0.06%, 0.03%, and 0.6%) in adult rats on immunoreactivity for ChAT protein in cholinergic areas. A – Immunoreactivity for choline acetyltransferase in the cholinergic nucleus of the medial septum (MS). B – Immunoreactivity for choline acetyltransferase in the basal cholinergic nucleus of Meynert (nbM). Values are expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by Fischer LSD post-test.

4. Discussion

In the present study, repeated exposure to clethodim (CL) induced a range of neurobehavioral, metabolic and neurochemical alterations in adult rats, indicating that even herbicides with a relatively short environmental half-life can elicit measurable biological effects following repeated exposure. Low-dose CL (0.06%) was sufficient to induce subtle motor alterations in the catalepsy test and to reduce spleen mass, whereas exposure to the highest concentration (0.6%) resulted in anxiety-like behavior and impaired working memory. Notably, independent of concentration, CL exposure consistently reduced food intake and increased depressive-like responses. Metabolic assessments further revealed that high-dose CL was associated with increased body mass and an altered glycemic profile. At the neurochemical level, CL exposure reduced hippocampal BDNF and c-Fos immunoreactivity at low and intermediate concentrations, while ChAT expression remained unchanged, suggesting that cholinergic synthetic capacity was preserved despite functional and molecular alterations.

Some neurotoxic compounds, such as rotenone and paraquat, are known to increase latency in the catalepsy test, reflecting impairments in motor initiation (Alqurashi et al., 2024; Ishola, 2018; Sanawar et al., 2022). Prolonged time spent on the bar is classically interpreted as a deficit in the initiation of voluntary movement (Santos et al., 2013). In contrast, in the present study, animals exposed to the lowest CL concentration (0.06%) exhibited a subtly reduced latency, which could not be associated with motor impairment.

In the forced swim test (FST), all CL-exposed groups exhibited increased immobility duration and a higher number of immobility episodes, while latency to the first immobility episode remained unchanged. This behavioral pattern is classically interpreted as a depressive-like phenotype, reflecting reduced motivational drive rather than motor impairment (Porsolt et al., 1979; Santos-Carrasco et al., 2025). Similar increases in immobility time have been consistently reported following exposure to pesticides from different chemical classes, including pyrethroids, organochlorines, and organophosphates (El Hamzaoui et al., 2024; Gargouri et al., 2019; Pinto Savall et al., 2021; Salama et al., 2019), suggesting that affective disturbances represent a common outcome of pesticide-induced neurotoxicity. Consequently, this may reflect compromised neurochemical functioning, potentially due to elevated oxidative stress (Santos-Carrasco et al., 2025).

Experimental evidence indicates that oxidative stress plays a central role in these behavioral alterations. For instance, melatonin administration attenuated depressive-like behavior induced by glyphosate exposure, an effect attributed to its antioxidant properties (El Hamzaoui et al., 2024). Likewise, chlorpyrifos exposure has been shown to induce depressive-like behavior across a range of doses, even in the absence of overt motor deficits (Chen et al., 2011; Ribeiro et al., 2022). In line with these observations, the present findings indicate that repeated CL exposure promotes depressive-like behavior independently of dose, reinforcing the notion that affective alterations may represent an early and sensitive indicator of pesticide-related neurotoxicity.

Previous studies have shown that oral administration of certain pyrethroid formulations does not induce acute anxiogenic effects in rats; however, chronic exposure over prolonged periods (e.g., 60 days) is sufficient to elicit anxiety-like behavior (Zhu et al., 2020). In contrast, in the present study, CL exposure induced anxiety-like behavior predominantly during the early phase of exposure, with no evidence of sustained anxiogenic effects following repeated administration. This transient behavioral response may be explained by the relatively short half-life of clethodim, estimated at approximately 2 hours (Li et al., 2025), which may limit its cumulative neurotoxic potential. Together, these findings suggest that CL-induced anxiety may reflect an acute neurobehavioral response rather than a progressive or persistent alteration.

Regarding short-term memory, animals exposed to CL exhibited a selective impairment in the spontaneous alternation (SA) task, as evidenced by a reduced number of correct alternations, while performance in the NOR test remained preserved. This dissociation likely reflects the distinct neural substrates underlying these tasks. SA primarily depends on working memory processes mediated by the hippocampal CA1 subfield and its interaction with the

prefrontal cortex, whereas NOR recruits a broader network involving hippocampal, perirhinal, entorhinal, and prefrontal regions, rendering it less sensitive to localized hippocampal dysfunction (Ennaceur and Delacour, 1988; Warburton and Brown, 2015).

Consistent with previous studies showing that pesticide exposure can impair working memory through oxidative stress–related mechanisms affecting cholinergic and hippocampal function (Aldridge et al., 2005; Bali et al., 2019; Icenogle et al., 2004), the present findings suggest that CL selectively disrupts CA1-dependent computations. Alterations in this hippocampal subfield are closely associated with deficits in working memory, given its central role in the rapid integration and transient storage of information (Basu and Siegelbaum, 2015; Khan et al., 2025). Accordingly, the reduced BDNF and c-Fos immunoreactivity observed in CA1 following CL exposure provides a neurochemical substrate for the working-memory deficits detected in the SA task, indicating impaired hippocampal plasticity and neuronal activation underlying short-term information processing.

Repeated exposure to the highest CL concentration (0.6%) induced a sustained increase in body mass beginning four days after the first exposure and persisting until day 48. This finding contrasts with previous studies reporting body mass reductions following CL exposure, even at high doses. For instance, Dcunha et al., (2023) observed decreased body mass only at the highest oral dose tested (200 mg/kg), while Abuzeid (et al., 2021) reported body mass reductions following sublethal oral administration (163 e 326 mg/kg). These discrepancies can be attributable to methodological differences, including route of exposure (nebulization versus oral gavage), formulation, dose range, and toxicokinetic profile.

More broadly, pesticide exposure has been associated with divergent effects on body mass, ranging from reductions to increases or no change, depending on the compound and exposure paradigm (Bhaskar and Mohanty, 2014; Gifford et al., 2015; Victor-Costa et al., 2010; Wang et al., 2013; Zhao et al., 2021). Notably, pesticide-induced weight gain has been linked to increased adiposity rather than lean mass, reflecting disruptions in lipid metabolism. Mechanistically, such effects may involve impaired adipocyte lipolysis, lipid peroxidation, altered fibroblast activity, and changes in gut microbiota composition, all of which contribute to increased fat accumulation (Heindel, 2019; Heindel et al., 2017; Miranda et al., 2023; Ren et al., 2020; Xiao et al., 2018). Thus, the body mass gain observed following high-dose CL exposure may reflect metabolic dysregulation rather than generalized toxicity.

Regarding food intake, all CL-exposed groups consumed less food than the control group. This effect may be related to to the endocrine-disrupting properties of several pesticides, which can increase leptin synthesis by adipocytes, thereby signaling the hypothalamus and

promoting satiety (Peris-Sampedro et al., 2015; Regnier et al., 2015; Sun et al., 2016). Nonetheless, the concomitant reduction in food intake and increase in body mass observed in CL-exposed animals indicates a more complex metabolic dysregulation rather than a simple consequence of reduced caloric intake, reinforcing the hypothesis that CL interferes with energy homeostasis and lipid metabolism.

In the glucose tolerance test, animals exposed to the highest CL concentration (0.6%) group exhibited its glycemic peak at only with 60 minutes post- gavage, whereas control and lower-dose Cl groups showed the expected peak at 30 minutes. According to the literature, delays in glycemic peaks may result from impaired gastric emptying or reduced intestinal absorption, both of which can limit the rapid availability of glucose in the bloodstream (Boaventura et al., 2023; Sala-Rabanal et al., 2018; Wielinga et al., 2005). Thus, the delayed glycemic response observed in the CL-0.6 group suggests that high concentrations CL exposure may interfere with gastrointestinal or metabolic regulatory mechanisms involved in glucose handling.

Previous studies have reported increases in organ mass following CL exposure administered via oral gavage, affecting the liver, kidneys, lungs, spleen, brain, and heart in rats (Abuzeid et al., 2021). In contrast, in the present study, we observed a reduction in spleen mass only in the CL-0.06 group. Similarly, Dcunha et al., (2023) demonstrated that oral CL administration in mice reduced kidney mass at all tested doses and decreased spleen and testicular mass at higher concentrations. These discrepancies likely reflect differences in exposure route, dosing regimen, and systemic bioavailability, suggesting that inhalational CL exposure may elicit subtler or more selective peripheral effects compared to oral administration.

c-Fos immunoreactivity was assessed to determin whether repeated CL exposure alters baseline neuronal activity in brain regions critically involved in memory processing. c-Fos is a well-established marker of neuronal activation and plays a key role in synaptic plasticity mechanisms underlying long-term potentiation and depression, which are essential for memory formation and refinement (Gandolfi et al., 2017; Giese, 2012; Khan et al., 2025). Our observations indicated that CL reduced neuronal activity at low and medium concentrations in the CA1 region, a subfield central to the acquisition, consolidation, and retrieval of memory (Khan et al., 2025). This decrease in activity could lead to memory deficits, as noted in the study by Liu et al., (2021). In their research, a mixture of paraquat and maneb was administered, resulting in memory deficits in the 8-arm radial maze, alongside reduced levels of c-Fos and BDNF in the hippocampus of rats.

Brain-derived neurotrophic factor (BDNF) is tightly linked to neuronal activity and synaptic plasticity, and several studies have demonstrated a positive association between BDNF expression and neuronal activation markers such as C-Fos (Alder et al., 2003; El-Sayed et al., 2011), although inverse or context-dependent relationships have also been reported (Imamura et al., 2005). In the present study, BDNF levels were reduced in the same hippocampal regions in which c-Fos immunoreactivity was decreased, whereas no significant changes were detected in other brain areas, including the mPFC, CA3, DG, MS, and nbM. This regional specificity suggests a selective vulnerability of hippocampal circuits to CL exposure.

Similar reductions in hippocampal BDNF have been reported following exposure to other agrochemicals, such as atrazine and organophosphates, and are consistently associated with memory deficits and depressive-like behaviors (Aliomrani et al., 2022; Dorri et al., 2015; Li et al., 2019b). Importantly, antioxidant co-treatments have been shown to restore BDNF levels in some of these models, implicating oxidative stress as a key upstream mechanism (Dorri et al., 2015). Given the central role of BDNF in promoting dendritic growth, synaptic maintenance, and neuroprotection (Chapleau et al., 2009; Colucci-D'amato et al., 2020), its reduction may compromise hippocampal plasticity and impair memory-related processes. Together, these findings support the notion that CL-induced reductions in BDNF, in parallel with decreased neuronal activation, represent a critical neurobiological substrate underlying the behavioral alterations observed in this study.

In the present study, CL exposure did not alter ChAT expression in major cholinergic nuclei, including the nucleus basalis magnocellularis (nbM) and te medial septum (MS), suggesting that chronic CL exposure does not induce sustained impairment of cholinergic synthesis. Given the short half-life of CL (Li et al., 2025), any acetylcholinesterase inhibition is likely transient and insufficient to promote long-term downregulation of ChAT expression. Although cholinergic projections from the MS and NBM critically modulate hippocampal function and memory processing (Müller and Remy, 2018; Swanson and Cowan, 1979), the preserved ChAT immunoreactivity observed here indicates that the behavioral and cognitive alterations induced by CL are more likely driven by disruptions in hippocampal neuronal activity and plasticity, rather than by persistent cholinergic dysfunction.

Taken together, the present findings indicate that the neurotoxic effects of CL are comparatively less severe than those reported for other widely studied pesticides, such as glyphosate and paraquat. Notably, this study represents the first investigation in rats combining behavioral assessment with immunohistochemical analyses of C-Fos, BDNF, and ChAT following CL exposure, which inherently imposes limitations related to the scarcity of

comparative data in the literature. The relatively modest neurotoxic profile observed may be partly attributable to the exposure regimen employed. At the time this study was designed, information regarding the toxicokinetics of CL was unavailable; subsequent evidence demonstrated that CL enantiomers exhibit a short half-life of approximately 2–3 hours (Li et al., 2025). In this context, future studies employing daily or continuous exposure paradigms will be essential to disentangle acute versus cumulative effects and to more fully characterize the neurobehavioral and metabolic consequences of CL exposure.

In conclusion, the present study demonstrates that repeated exposure to CL, even at low and moderate concentrations, induces measurable neurobehavioral, metabolic, and neurochemical alterations in rats. Impairments in working memory, the emergence of depressive-like behavior, changes in body mass, and reductions in hippocampal BDNF and c-Fos expression collectively indicate that CL may interfere with neuronal activity and synaptic plasticity in brain regions critically involved in cognition and emotional regulation. Although the short half-life of CL may limit the persistence of its effects, these findings highlight that this herbicide is not biologically inert to mammals and may pose underestimated risks, under repeated exposure conditions. Future studies incorporating daily exposure regimens, time-resolved neurochemical analyses, and evaluations of commercial formulations are needed to better elucidate the mechanisms and potential health implications of chronic CL exposure.

Author Contributions

A.J.B., C.E.J.L., J.L.S., A.C.L., M.S.M., M.C.S.O., R.J.O.F., G.S.R. and K.A.A.L.M. contributed to data acquisition, animal experimentation, and behavioral analyses; J.C.J.S.L., H.F.S., L.L.C.R.L. and M.A.M.F. performed biochemical and neurochemical analyses and assisted with data interpretation; A.M.G. and J.R.S. contributed to the conceptualization and design of the study; A.M.G., J.R.S. and K.A.A.L.M. conducted the statistical analysis; A.J.B., C.E.J.L. and J.R.S. drafted the original manuscript; A.M.G. M.A.M.F., H.F.S. and J.R.S. critically revised the manuscript for important intellectual content; J.R.S. supervised the study, secured funding, and is responsible for the final approval of the version to be published. All authors read and approved the final manuscript.

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Conflicts of interest

The authors report no conflicts of interest.

Data Availability Statement

The data that support the findings of this study areavailable from the corresponding author upon reason-able request.

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5. **CONCLUSÃO**

Nosso trabalho demonstrou que a exposição crônica ao CL foi capaz de induzir comportamento do tipo ansioso de forma aguda, depressivo de forma crônica, déficit de memória de curto prazo, aumento da massa corporal mesmo após uma redução da ingestão alimentar, redução da massa do baço e atraso do pico glicêmico. Além disso, observamos uma redução da imunorreatividade de c-Fos e de BDNF no hipocampo, mas sem alterações de ChAT em regiões colinérgicas.

Esses achados sugerem que os efeitos comportamentais e emocionais induzidos por CL podem se relacionar à redução da ativação neuronal e da sinalização de BDNF hipocampal, sem influência primária detectável do sistema colinérgico nas regiões analisadas. Novos estudos devem ser realizados para melhorar a compreensão dos mecanismos subjacentes aos achados aqui demonstrados.

6. **PERSPECTIVAS:**

Nosso trabalho utilizou uma metodologia com exposição repetida de CL, a cada 48 horas, ao longo de 50 dias. Entretanto, faz-se necessário novos trabalhos que avaliem os efeitos do CL de forma aguda nas tarefas comportamentais, pois a meia vida desse composto e seus enantiômeros é de apenas 2 h. Além disso, abrimos lacunas para que novas investigações possam ser realizadas para compreender melhor sobre as possíveis alterações metabólicas e inflamatórias relacionadas ao aumento da massa corporal, atraso do pico glicêmico e redução da massa do baço. Seria o CL uma droga causadora de síndrome metabólica?

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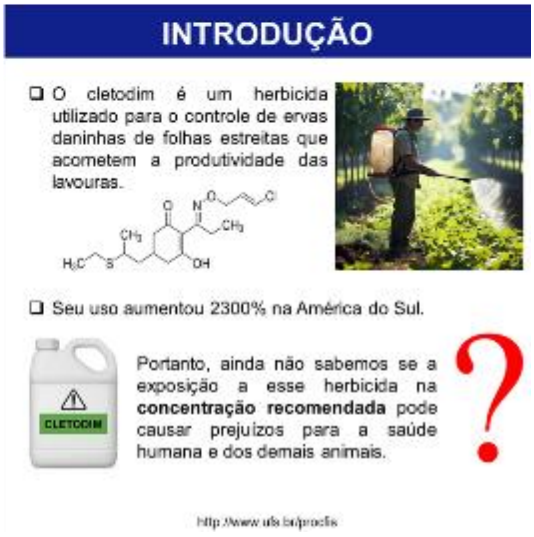
APÊNDICE I

MATERIAL PARA DIVULGAÇÃO



OBJETIVOS

❑**Objetivo Geral:**

- Avaliar as alterações comportamentais e fisiológicas após exposição repetida de cletodim em ratos

❑**Objetivos específicos:**

- Investigar as alterações:
  - Comportamentais, motoras e cognitivas;
  - Quanto a ingestão alimentar, massa corporal, dos órgãos e tolerância glicêmica;
  - Sobre a imunorreatividade das proteínas BDNF, c-Fos e ChAT em áreas encefálicas.
- Após a exposição repetida de cletodim em ratos.

http://www.ufs.br/procfis

METODOLOGIA

❑ Possui aprovação do CEUA/UFS (nº 5228100323).

❑ Foram utilizados 30 *Rattus norvergicus* machos adultos da linhagem Wistar.

❑ Divididos em 4 grupos para serem expostos via através da nebulização.

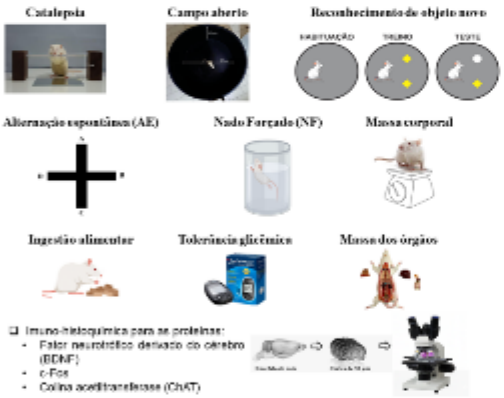
25 nebulizações
1 a cada 48 horas

- Grupo controle (CTL, n = 7) → Soro fisiológico;
- Grupos Cletodim (CL):
  - CL-0,06 (n = 7) → CL a 0,06%, concentração recomendada;
  - CL-0,3 (n = 8) → CL a 0,3%, cinco vezes mais concentrada;
  - CL-0,6 (n = 8) → CL a 0,6%, dez vezes mais concentrada.

http://www.ufs.br/procfis

METODOLOGIA

❑ Ao longo dos 50 dias de experimento, os animais foram submetidos aos testes comportamentais e avaliações:



❑ Imuno-histoquímica para as proteínas:
- Fator neurotrófico derivado do cérebro (BDNF)
- c-Fos
- Colina acetiltransferase (ChAT)

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RESULTADOS

Grupo CTL  Normal	CL-0,06 	CL-0,3 	CL-0,6 
Comportamento motor	Sem alterações	Sem alterações	Sem alterações
Comportamento ansioso	Sem alterações	Sem alterações	Menor tempo no centro
Memória operacional	Sem alterações	Sem alterações	Menor número de acertos
Imobilidade no TNF	Maior tempo imóvel	Maior tempo imóvel	Maior tempo imóvel
Massa corporal	Sem alterações	Sem alterações	Aumento na massa corpórea
Tolerância glicêmica	Sem alterações	Sem alterações	Atraso para atingir um pico glicêmico
Ingestão alimentar	Reduzida	Reduzida	Reduzida
BDNF e c-Fos (CA1, CA3, GD e CPFm)	Imunoreatividade reduzida em CA1	Imunoreatividade reduzida em CA1	Sem alterações
ChAT (nbM e SM)	Sem alterações	Sem alterações	Sem alterações

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CONCLUSÃO

O cletodim, mesmo na concentração recomendada é capaz de causar prejuízos aos animais expostos. Esses achados reforçam a importância do uso de EPI pela população, assim como, incentivar os agricultores a utilizarem a concentração recomendada pelo fabricante, pois percebemos maior número de alterações na concentração mais elevada.

ANEXO I

Universidade Federal de Sergipe

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "ALTERAÇÕES COMPORTAMENTAIS E MONOAMINÉRGICAS APÓS EXPOSIÇÃO REPETIDA DE CLETHODIM EM RATOS", protocolada sob o CEUA nº 5228100323 (ID 000852), sob a responsabilidade de **José Ronaldo dos Santos** e *equipe; Abraão de Jesus Barbosa; Auderlan Mendonça de Gois; Milena Caroline Nunes Monteiro; José Leandro Santos Souza; Ana Cleia Alves Da Luz; Mylaine Santos Mendonça; Heitor Franco Santos; João Eduardo Conceição Melo; Lívia Cristina Rodrigues Ferreira Lins* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Sergipe (CEUA/UFS) na reunião de 22/03/2023.

We certify that the proposal "BEHAVIORAL AND MONOAMINERGIC CHANGES AFTER REPEATED EXPOSURE TO CLETHODIM IN RATS", utilizing 32 Heterogenics rats (32 males), protocol number CEUA 5228100323 (ID 000852), under the responsibility of **José Ronaldo dos Santos** *and team; Abraão de Jesus Barbosa; Auderlan Mendonça de Gois; Milena Caroline Nunes Monteiro; José Leandro Santos Souza; Ana Cleia Alves Da Luz; Mylaine Santos Mendonça; Heitor Franco Santos; João Eduardo Conceição Melo; Lívia Cristina Rodrigues Ferreira Lins* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Sergipe (CEUA/UFS) in the meeting of 03/22/2023.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 05/2023 a 05/2024 Área: Fisiologia

Origem:	Biotério da Universidade Federal de Sergipe						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	6 a 8 meses	N:	32
Linhagem:	Rattus novergicus/Wistar			Peso:	350 a 450 g		

Local do experimento: Os animais estarão alocados no Laboratório de Neurofisiologia (LNFS) durante realização dos experimentos, sendo o biotério de apoio o Biotério setorial, ambos localizados no departamento de fisiologia.

São Cristóvão, 29 de março de 2023

Prof. Dr. Josemar Sena Batista
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Sergipe

Prof. Dr. Anderson Carlos Marçal
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Sergipe