PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

RAÍSSA DE OLIVEIRA MANTOVANI

ANÁLISE MORFOFUNCIONAL CARDÍACA EM RATOS WISTAR SUBMETIDOS A EXPOSIÇÃO CRÔNICA AO HERBICIDA GLIFOSATO; TRADUÇÃO, ADAPTAÇÃO CULTURAL E AVALIAÇÃO DE CONCORDÂNCIA DA FERRAMENTA SYRCLE-Rob PARA O PORTUGUÊS BRASILEIRO

Presidente Prudente - SP
2024

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Tese apresentada Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutor – Área de concentração: Fisiopatologia Animal

Orientadora: Prof[a]. Dra Francis Lopes Pacagnelli

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Presidente Prudente, 26 de Setembro de 2024.

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DEDICATÓRIA

Aos meus pais, Rita e Sidnei que se dedicaram e me apoiaram ao máximo para que eu conquistasse todos meus sonho.

Aos meus avós Alice e Leonardo (in memorian) que do céu sempre olharam por mim.

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“Não há nada a temer na vida, apenas tratar de compreender.”

(Marie Curie)

RESUMO

Análise morfofuncional cardíaca em ratos wistar submetidos a exposição crônica ao herbicida glifosato; tradução, adaptação cultural e avaliação de concordância da ferramenta SYRCLE-Rob para o português brasileiro

Essa tese foi composta por dois artigos. O primeiro artigo foi intitulado: A exposição inalatória crônica em alta dose ao herbicida glifosato impacta em remodelamento cardíaco sem repercussão funcional em ratos, com o objetivo de avaliar os efeitos de diferentes concentrações de glifosato em corações de ratos submetidos à exposição inalatória crônica. Assim quarenta e dois ratos Wistar foram divididos em três grupos experimentais (n=14 animais/grupo) e expostos à inalação crônica de diferentes concentrações de glifosato durante 180 dias (segunda a sexta-feira). O grupo controle (C) foi submetido à nebulização com 10 ml de água destilada, enquanto o grupo de baixa concentração (LC) foi exposto a $3{,}71x10^{-3}$ gramas de princípio ativo por hectare (g.i.a./ha) e o grupo de alta concentração (HC) a $9{,}28x10^{-3}$ g.i.a./ha. Ao final do experimento, foi realizada ecocardiografia para avaliação da função cardíaca. Posteriormente, os animais foram eutanasiados e os corações dissecados para análise histológica do ventrículo esquerdo, incluindo a avaliação de fibrose, dimensão fractal, marcadores de inflamação e apoptose, além de biomarcadores do estresse oxidativo. A análise ecocardiográfica não revelou alterações funcionais significativas entre os grupos. Da mesma forma, as análises anatômicas e histomorfométricas não evidenciaram modificações estruturais e a razão BAX/BCL-2, indicativa de apoptose, permaneceu inalterada. Entretanto, observou-se aumento significativo de colágeno no grupo HC em relação ao C, além de uma redução na citocina pró-inflamatória TNF-α nos grupos LC e HC em relação ao C. Houve também aumento da atividade da enzima antioxidante CAT e redução da GST no grupo HC em relação ao C. Conclui-se que a exposição inalatória crônica ao herbicida glifosato resultou em remodelação cardíaca caracterizada pelo aumento do colágeno com resposta adaptativa protetora sem prejuízo funcionais. O segundo artigo foi intitulado: Tradução, adaptação cultural e avaliação de concordância da ferramenta SYRCLE-rob para o português, em que o objetivo foi traduzir, adaptar e validar a ferramenta SYRCLE-RoB para o português brasileiro. A tradução da ferramenta foi realizada seguindo guia internacional. Após tradução, retrotradução e

aprovação da versão pelos criadores, a ferramenta, em português, foi avaliada quanto à concordância. Para isso, foram escolhidos aleatoriamente onze artigos envolvendo animais de experimentação, que contavam com intervenção e em inglês, após consulta ao PubMed. A versão traduzida e adaptada do SYRCLE-RoB foi aplicada em cada estudo por 2 pesquisadores. Considerando que o SYRCLE-RoB contém 10 itens de análise e foram utilizados 11 artigos para avaliação, calculamos o percentual de concordância em 111 condições. A concordância total foi de 53,33% (Kappa de Cohen= 0,18) considerando todos os itens do instrumento. A taxa média de concordância para os itens 1 a 7 foi de 76,19% (Kappa de Cohen = 0,47). O instrumento SYRCLE-RoB traduzido para o português brasileiro apresentou confiabilidade moderada, o que pode ser considerado válido para aplicação em português como forma de avaliação metodológica em estudos pré-clínicos.

Palavras-chave: glifosato; remodelação cardíaca; riscos de viés; qualidade metodológica; modelo animal.

ABSTRACT

This thesis was composed of two articles. The first article was entitled: Chronic high-dose inhalation exposure to the herbicide glyphosate affects cardiac remodeling without functional repercussions in rats, with the aim of evaluating the effects of different concentration of glyphosate on rat hearts following chronic inhalation exposure. So forty-two Wistar rats were exposed via inhalation and divided into three experimental groups (n=14 animals/group): a control group (C) exposed to nebulization with 10 ml of distilled water; a low concentration group (LC) exposed to 3.71x10−3 grams of active ingredient per hectare (g.i.a./ha); and a high concentration group (HC) exposed to 9.28x10−3 g.i.a./ha. The animals were exposed for 180 days (Monday to Friday). After the final exposure, echocardiography was performed to assess cardiac function, and then the rats were euthanized. The heart was dissected, and the left ventricle was analyzed for histological changes, fibrosis, fractal dimension, immunohistochemical analysis for inflammation and apoptotic pathways, and oxidative stress biomarkers. Echocardiographic analysis did not reveal significant functional differences between the groups. Similarly, anatomical and histomorphometric analyses showed no structural modifications, and the BAX/BCL-2 ratio, indicative of apoptosis, remained unchanged. However, a significant increase in collagen was observed in the HC group compared to the C group, along with a reduction in the pro-inflammatory cytokine TNF-α in both the LC and HC groups compared to the C group. Additionally, there was an increase in the activity of the antioxidant enzyme CAT and a reduction in GST the HC group compared to the C group. It is concluded that chronic inhalation exposure to the herbicide glyphosate resulted in cardiac remodeling characterized by increased collagen with a protective adaptive response, without functional impairment. The second article was titled: Translation, cultural adaptation, and concordance evaluation of the syrcle-rob tool into brazilian portuguese, where the aim was to translate, adapt and validate the SYRCLE-RoB tool into Brazilian Portuguese. The translation of the tool was carried out following an international guide. After translation, back-translation and approval of the version by the creators, the tool, in Portuguese, was evaluated for agreement. For this, eleven articles involving experimental animals, which had intervention and in English were randomly chosen after consulting PubMed. The translated and adapted version of SYRCLE-RoB was applied in each study by 2 researchers. Statistical

analysis: To analyze agreement between evaluators, the Fleiss kappa index was calculated for each item. Considering that SYRCLE-RoB contains 10 analysis items and 11 articles were used to evaluate, we calculated the percentage of agreement in 111 conditions. Total agreement was 53.33% (Cohen's Kappa= 0.18) considering all items in the tool. The average agreement rate for items 1-7 was 76.19% (Cohen's Kappa = 0.47). The SYRCLE-RoB instrument translated into Brazilian Portuguese showed moderate reliability, which can be considered valid for application in Portuguese as a form of methodological evaluation in pre-clinical studies.

Keywords: glyphosate; cardiac remodeling; risk of bias; methodological quality; animal studies.

LISTA DE SIGLAS

2,4-D	2,4-dichlorophenoxyacetic acid
EPSPS	5-enolpyruvate-shikimate-3-phosphate synthase
g.i.a./ ha	Active ingredient per hectare
ATR	Atrazine
ATS	Atria
BWT	Back wall thickness
BAX	B-cell CLL/lymphoma2
BCL-2	Bcl2-associated X protein
PCO	Carbonylated protein
CFs	Cardiac fibroblastos
CAT	Catalase
C	Control
E/A	E wave/A wave
EF	Ejection fraction
CEUA	Ethics Committee on the Use of Animals
ECM	Extracellular matrix proteins
FRAP	Ferric Reducing Antioxidant Power
FBW	Final body weight
GST	Glutathione S-transferase
g	Grams
HR	Heart rate
HE	Hematoxylin and Eosin
HC	High concentration
IARC	International Agency for Research on Cancer
LV	Left ventricle
LVDD	Left ventricular diastolic diameter
TRIV	Left ventricular isovolumetric relaxation time
LVSD	Left ventricular systolic diameter
LPO	Lipids by oxidation
LC	Low concentration
MDA	Malondialdehyde
NBT	Nitroblue-tetrazolium
NF-kB	Nuclear factor kappa B

% ENC END	Percentage of endocardial shortening
PSR	Picrosirius
PWT	Posterior wall thickness
GSH	Reduced glutathione
AT/FBV	Relationship between atria and final body weight
LV/FBV	Relationship between left ventricle and final body weight
RV/FBV	Relationship between right ventricle and final body weight
RV	Right ventricle
SBW	Starting body weight
SOD	Superoxide dismutase
NADPH	Superoxide radicals
TBARS	Thiobarbituric acid
GT	Thoral glutathione
TNF- α	Tumor necrosis factor alpha
EPA	United States Environmental Protection Agency

SUMÁRIO

1 ARTIGO CIENTÍFICO 1

Chronic high-dose inhalation exposure to the herbicide glyphosate affects cardiac remodeling without functional repercussions in rats

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LIFE SCIENCES - Qualis Capes A1 – Fator de Impacto 5.2

ABSTRACT

Aim: Glyphosate is a widely used herbicide in agriculture, primarily acting by inhibiting weed growth. The main route of exposure to glyphosate is through inhalation during its application, which can lead to significant cardiac functional damage**.** We evaluated the effects of different concentrations of glyphosate on rat hearts following chronic inhalation exposure. **Material and methods:** Forty-two Wistar rats were exposed via inhalation and divided into three experimental groups (n=14 animals/group): a control group (C) exposed to nebulization with 10 ml of distilled water, a low concentration group (LC) exposed to 3.71x10−3 grams of active ingredient per hectare (g.i.a./ha), and a high concentration group (HC) exposed to 9.28x10−3 g.i.a./há, for 180 days (Monday to Friday). After the final exposure, echocardiography was performed to assess cardiac function, and then the rats were euthanized. The heart was dissected, and the left ventricle was analyzed for histological changes, fibrosis, fractal dimension, and oxidative stress biomarkers, and immunohistochemical analysis was performed to evaluate inflammation and apoptotic pathways. **Key findings:** Echocardiographic analysis did not reveal significant functional differences between the groups. Similarly, anatomical and histomorphometric analyses showed no structural modifications, and the BAX/BCL-2 ratio, indicative of apoptosis, remained unchanged. However, a significant increase in collagen was observed in the HC group compared to the C group, along with a reduction in the pro-inflammatory cytokine TNF-α in both the LC and HC groups compared to the C group. Additionally, there was an increase in the activity of the antioxidant enzyme CAT and a reduction in GST the HC group compared to the C group. **Significance:** It is concluded that chronic inhalation exposure to the herbicide glyphosate resulted in cardiac remodeling characterized by increased collagen with a protective adaptive response, without functional impairment.

Keywords: 1. Glyphosate. 2. Heart 3. Pesticides. 4. Antioxidant system.

1. Introduction

Recent data on the global production and use of pesticides revealed an increase of approximately 3.5 million tons by 2020, with more than 70% of the total output being used in agriculture for pest control (1). Glyphosate (N-phosphonomethylglycine), the active ingredient in the herbicide Roundup®, is an organophosphate compound, and is considered one of the most widely used herbicides, accounting for 60% of the world market (2,3). It acts as an inhibitor of the enzyme EPSPS (5-enolpyruvate-shikimate-3-phosphate synthase) of the shikimic acid metabolic pathway, preventing the synthesis of certain amino acids that are essential for plant growth, and having high efficiency in eliminating weeds(4,5). The occupacional exposure by inhalation is responsible for the most cases of intoxication on farm workers (1).

Several international organizations have reported and investigated the impacts of glyphosate. In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans"(6), based on limited human evidence but sufficient evidence from animal studies, concluding that glyphosate is also genotoxic and triggers oxidative stress (7). On the other hand, the U.S. Environmental Protection Agency (EPA) concluded that glyphosate is not carcinogenic to humans, highlighting the controversy and complexity of studies regarding the safety of this herbicide (8).

Regarding the cardiovascular system, a retrospective study of 153 patients who ingested glyphosate-surfactant reported abnormal electrocardiographic alterations immediately after consumption, including QTc interval prolongation, followed by intraventricular conduction delay and first-degree atrioventricular block (9). It has also been reported a caso of intoxication in a paciente on was reported in a patient who used glyphosate (50%) in her garden for weeks without any protection, resulting in syncope and electrocardiogram (ECG) alterations with a left bundle branch block (10). In rats, the cardiovascular system was evaluated subchronically over 75 days via inhalation of the same dosage as used in the current study, showing that glyphosate has atherogenic potential (11). Further, structural abnormalities in the atrium and ventricle, irregular heart rhythm, situs inversus, and decreased heartbeats were observed in zebrafish treated with GBH during heart development treated though immersion of embryos for 48 hours with 50 g/ml of GBH, starting at gastrulation (12).

Also, in experimental animals, exposure to xenobiotics often induces oxidative stress, which can cause significant metabolic and functional changes in tissues and trigger

inflammatory processes, creating a cycle in which inflammation can further generate oxidative stress and fibrosis, as has been demonstrated in the liver, kidneys, and lungs (13–16). However, the long-term evaluation of the inflammatory profile, oxidative stress, and structural alterations in the cardiovascular system has not yet been elucidated. These evaluations are crucial as long-term exposure to glyphosate-based herbicides can directly influence cardiac functionality and predict severe cardiovascular events. Therefore, in the present study, we evaluated the effects of different concentrations of a glyphosate-based herbicide on the hearts of rats subjected to chronic inhalation exposure.

2. Materials and methods

This study was approved by the Animal Use Ethics Committee of the University of Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil (Protocol 5864). It also complies with the principles of laboratory animal care formulated by the Brazilian College of Animal Experimentation (COBEA). Additionally, we followed the ARRIVE recommendations.

2.1. Experimental design

Forty-two adult male albino Wistar rats (300-450g) were supplied by the UNOESTE Central Vivarium and housed at the UNOESTE Experimental Vivarium. The animals were allocated in collective plastic cages (5 animals per cage and one cage with 4 animals), measuring 30 cm × 16 cm × 19 cm, at a controlled temperature (22 ± 2 °C), relative humidity of 50 ± 15%, with cycles of 12 hours of light (light period between 7 a.m. and 7 p.m., dark period between 7 p.m. and 7 a.m.). The animals received a standard diet for laboratory animals (Primor®) and water ad libitum.

Exposure to the glyphosate herbicide was conducted using Glyphosate [N-(phosphonomethyl) glycine] (Roundup Original DI, Monsanto, São Paulo, Brazil), registered with the Ministry of Agriculture, Livestock, and Food Supply – MAPA – under No. 00513, concentration: Glyphosate diammonium salt 445 g/L (370 g/L acid equivalent). Doses were adapted to the box area in order to simulate environmental exposure (17).

The exposure protocol involved two boxes (32 x 24 x 32 cm), each connected to an ultrasonic nebulizer from Pulmosonic Star. All groups were exposed for approximately 15 minutes daily, from Monday to Friday, for a period of 180 days, with 14 animals assigned to each group.

The animals were randomly divided by lot into the following three groups (n = 42): Inhalation Control (CG) (n = 14): Exposed to nebulization with a solution containing 10 ml of distilled water; Low Concentration (LC) (n = 14): Exposed to herbicide fogging with 3.71×10^{-3} g of active ingredient per hectare (g.i.a./ha) (4.6 µl of the pesticide added to distilled water), corresponding to 187.17 mg/m³ of glyphosate; High Concentration (HC) (n = 14): Exposed to herbicide fogging with 9.28×10^{-3} g.i.a./ha (11.5 µl of pesticide added to distilled water), corresponding to 467.93 mg/m³ of glyphosate (18)

The different concentrations of the herbicide were diluted in 10 ml of distilled water to perform the nebulization. The solutions were prepared at the time of use. The different concentrations of the glyphosate herbicide were formulated based on the product label, which shows the different herbicide concentrations for each type of crop to be sprayed, and a dose-adjustment was made to the box area to simulate environmental occupational exposure (19).

All animals were exposed for 6 months, and after this period, they were euthanized (17). Anesthesia and euthanasia were performed with thiopental sodium (Syntec, USA) at doses of 40 mg/kg and 100 mg/kg, respectively, administered into the peritoneal cavity. The indications of death were the absence of respiratory movements, and heartbeat, and loss of reflexes (20). The experimental design is provided in Fig.1.

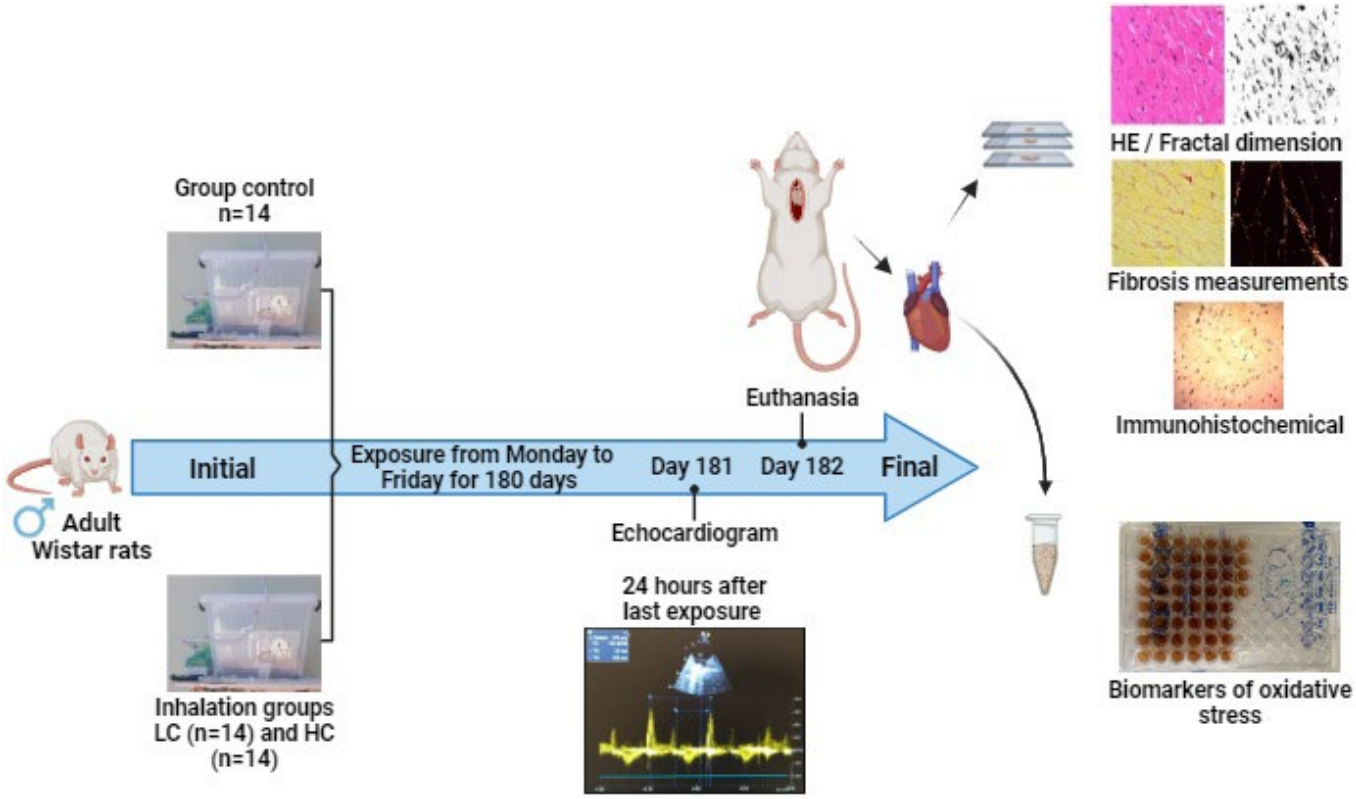

Fig. 1. Experimental design. Created in Biorender.

2.2. Echocardiogram

Twenty-four hours after the last nebulization, an echocardiographic evaluation was performed using a commercially available echocardiograph (General Electric Medical Systems, Vivid T8 Pro, Israel) equipped with a 5 to 12 MHz multifrequency probe. Rats were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg, IP, Dopalen®) and xylazine (0.5 mg/kg, IP, Anasedan®). A 2-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral valve leaflets, and at the level of the aortic valve and left atrium. M-mode images of the LV were recorded on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV tracings were manually measured by the same observer. The measurements obtained were the mean of at least 5 cardiac cycles on the M-mode tracings. Heart rate (HR) was assessed, and the following structural variables were measured: left ventricular diastolic and systolic dimensions (LVDD and LVSD, respectively), left ventricular diastolic posterior wall thickness (PWT), and LV diastolic septal wall thickness (SWT). LV mass was calculated using the formula $[(LVDD + PWT + SWT)^3 - (LVDD)^3] \times 1.04$. Relative LV wall thickness (LV Mass Index) was calculated using the formula $2 \times PWT/LVDD$. LV systolic function was calculated using the following parameters: % endo shortening (EEP), % mesocardial

shortening (MEP), and velocity of posterior wall shortening of LV (% Endo. Short). LV diastolic function was assessed by early and late diastolic mitral inlet velocity (E and A waves), E/A ratio, and isovolumic relaxation time (IVRT). A joint assessment of LV diastolic and systolic function was performed using the myocardial performance index (Tei Index), also known as the myocardial performance index, calculated as follows: [(Tei-a (isovolumic contraction time + ejection time + isovolumic relaxation time) – Tei-b (isovolumic contraction time + ejection time + isovolumic relaxation time) ÷ Tei-b)]. The study was complemented with evaluation by tissue Doppler image of the systolic velocity (TDIS'), early diastolic velocity (E') and late diastolic velocity (A') of the mitral annulus (mean velocities of the lateral and septal walls) (21–23).

2.3. Analysis of anatomical parameters

After the experimental period, the rats were weighed (FBW), euthanized, and the heart was dissected into the left ventricle (LV), atria (ATs) and right ventricle (RV) and then weighed. The LV/FBW, AT/FBW and RV/FBW ratio were used as a hypertrophy index (18)

2.4. Histological analysis

Cardiac tissue samples were fixed in a 10% buffered formalin solution for a period of 48 hours. After fixation, the tissues were stored in paraffin blocks, which allowed coronal histological analysis of the 4 μm sections. The histological sections were stained on a slide with Hematoxylin-Eosin (HE) solution to measure the cross-sectional areas of the cardiomyocytes, a LEICA microscope (DM750 model, Germany) was used, which sent the digital images to the computer using the Leica Application Suite LAS 4.2.0 image analysis system (Media Cybernetics, Silver Spring, Maryland, USA) (24–26). The images were obtained by optical microscope binoculars. All images were captured by a video camera at 400x magnification (40x objective). Four sections of the left ventricle will be obtained from each animal in different fields, analyzing the chosen captures according to the place where more cells can be seen in a cross-section (24,26,27). A quantitative analysis was performed to evaluate cells in apoptosis, necrosis, fibrosis, and inflammation (28–31)

2.5. Fractal dimension

The fractal dimension of the tissue was evaluated in both sections of the left ventricle stained with Picrosirius Red (PSR) and Hematoxylin-Eosin (HE). For the fractal dimension, three images of each animal were photographed, with a magnification of 400× to evaluate

nuclear regularity in HE or fibrosis in PSR. The fractal dimension was estimated by the box counting method. This was aided by ImageJ® software from the US National Institutes of Health (NIH). It is available free of charge on the Internet (http://rsbweb.nih.gov/ij/). This software uses the box-counting method as a two-dimensional approach, allowing the quantification of the distribution of pixels in a specific space. However, this method does not analyze the texture of the image. With this method, two images with the same pixel distribution (one binarized and one grayscale) have the same fractal dimension. Therefore, the fractal dimension calculated with ImageJ® is always between 0 and 2 (32).

2.6. Fibrosis measurements

Fibrosis measurements of left ventricle tissue samples were fixed in 10% of buffered formalin solution for a period of 48 h. After fixation, the tissues were stored in paraffin blocks, which allowed for the coronal histological analysis of the 4-µm sections. The collected samples were stained with Picrosirius red (PSR) according to the standard protocol of the laboratory. Cardiac sections stained with PSR were used to quantify fibrosis; Analysis was performed using ImageJ following software instructions for collagen quantification (33). Picrosirius staining viewed under polarized light enables the differentiation of type I (red) and type III (green) collagen. We used Image J software to measure the medium of coloration of these collagens in relation to the total image area (34).

2.7. Immunohistochemical analysis and quantification of the expression of inflammatory markers, apoptotic index of the hearts

Cardiac muscle samples from five animals each group were used for immunostaining. The left ventricle section was subjected to peroxidase and protein blocks. In the next step, the sections were subjected to reaction with specific primary antibodies with the inflammatory markers TNF-α (52B83, sc-52746) (1:100), NF-κB (E-10, sc-8414) (1:100), and with the apoptotic markers BAX (B-9, sc-7480) (1:300), BCL-2 (C-2, sc-7382) (1:300) mouse monoclonal IgG, and incubated in a humid chamber overnight. The samples were incubated with a secondary monoclonal antibody, m-IgGk HRP (sc-516102), at room temperature, developed with diaminobenzidine (DAB), stained with Harris hematoxylin 20%. Ten images of the five rats from each group were acquired using the Zeiss Axiophoto (Zeiss, Munich, Germany) photomicroscope at 400x magnification and quantified using ImageJ® software (version 1.5). They were quantified by the percentage of their area.

2.8. Biomarkers of oxidative stress

The left ventricle was homogenized in 1 ml of phosphate buffer (pH 7.4) and centrifuged at 9500 g for 10 min at 4°C. The protein quantification of the samples was determined by the (35) method. Samples were then normalized to 1 mg/mg protein and used for the following analyses. The analysis of the oxidative profile will be performed through the quantification of lipid peroxidation (LPO) and other antioxidant substances.

2.9. Lipid peroxidation (LPO)

The LPO was measured to indirectly quantify the peroxides produced. The result reflects the intensity of LPO (36). Measurements were performed using the method of reactive substances to thiobarbituric acid (TBARS) with an absorbance of 535 nm and 572 nm (37) compared to the standard curve for malondialdehyde (MDA), the main by-product of cellular LPO. To prepare the test, 50 µl of each normalized sample was pipetted in duplicate in a microplate, followed by the addition of FeCl3 (1M), ascorbic Shaked and placed in a water bath at 90 °C for 15 min. The plate was then cooled to stop the reaction, and then read at 535 and 572 nm.

3.0. Carbonylated protein quantification (PCO)

For the quantification of carbonylated proteins, we used the method described by Reznick and Packer (38) with adaptations. For so, the samples were homogenized and normalized for 1 mg of protein mL-1. In microtubules, we added 300 uL of the homogenates and added either 500 uL of HCl 2M or reaction medium (DTNB 0,1982% diluted in HCl 2M). Reactions were incubated for an hour with vigorous agitation every 15 minutes. After that, 500 uL of TCA 28% was added in all tubules and this reaction was incubated for 10 minutes for protein precipitation. Then, the tubules were centrifuged at 9.000g for 10 minutes and the supernatant was disposed of. The pallet formed was resuspended in 1 mL of ethanol and ethyl acetate (1:1), then, the tubules were centrifuged again under the same conditions and the supernatant was also disposed of. This washing process was repeated for 2 more times. Then, 350uL of guanidine hydrochloride 57,31% was added to the tubule and the reaction was incubated for 10 minutes. Then, 150uL of this final solution was transfered into a 96 -well microplate in duplicate and the absorbance was read at 380 nm.

3.1. Reduced glutathione (GSH)

Reduced glutathione (GSH) levels were determined as proposed by Rahman et al. (39) with some modifications. For this, 5,5-dithiobis (2-nitrobenzoic acid) NBT was used in the left ventricle homogenate supernatant and evidenced by a yellow color formation. GSH levels were measured at 412 nm and results were expressed as micromoles/mg protein.

3.2. Catalase (CAT) activity

The enzymatic activity of CAT was determined by the degradation of hydrogen peroxide into oxygen and water. After determining the protein concentration (normalized 1.0 mg/ml in PBS), 297 µl of the reaction medium was placed in a UV4 microplate (in triplicate) at 240 nm for 60 s (40).

3.3. Superoxide dismutase (SOD) activity

The evaluation of the activity of the enzyme SOD was performed as described by Senthilkumar et al. (41) with some changes. The enzyme comes from homogenates normalized to 1 mg/ml. A reaction mixture was prepared to contain sodium carbonate buffer (50 mM, pH 10.2), nitro blue tetrazolium (NBT) (96 uM) and Triton X-100 (0.6%), which was incubated for 2 min with sodium hydrochloride. hydroxylamine (NH2OH·HCl) (20 mM, pH 6.0). The final volume was adjusted to 200 µl. The reaction consists of the quantification of complexes formed by superoxide anions with the addition of NBT and NH2OH·HCl of yellowish color with the reduction of NBT, forming a bluish color read at 560 nm for 2 min at intervals of 15 s (42)

3.4. Glutathione transferase (GST) activity

The enzymatic activity of glutathione S-transferase (GST - EC 2.5.1.18) of the left ventricle as determined through the formation of a thioether from the interaction of GSH with CDNB, the increase in absorbance through the formation of the thioether was monitored at 340 nm (RS: 100mM potassium phosphate buffer pH 6.5; 1.5mM GSH; 2mM CDNB) for 5 min at 40 s intervals, as described by Keen et al. (43) Values were expressed in µM Thioether formed min/mg/protein.

3.5. Total glutathione concentration

GT concentration will be determined using 5,5'-thiobis 20-nitrobenzoic acid (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR) on

the sample. GT concentrations will be measured at 412 nm after 15 minutes of incubation of samples with the reaction medium (39).

3.6. Concentration of antioxidants by the FRAP method

To evaluate the iron reduction power, the method of Benzi and Strain (44) with adaptations is used. For this, the FRAP solution will be prepared by adding 25.0 mL of 0.3 M acetate buffer to 2.50 mL of 20.0 mM ferric chloride hexahydrate and 2.50 mL of 10.0 mM TPTZ. For the test, samples already normalized (1 mg of protein per mL of sample) will be used. In a microplate, 150 ul of FRAP reagent will be added to 50 ul of the sample. The reading will be taken immediately after the addition of the reagent, at 595 nm. The antioxidant potential of the tissues will be determined based on a calibration curve, traced using Trolox in concentrations that varied between 0.0325 mM and 0.5 mM.

3.7. Data analysis

Data normality was assessed by the Shapiro-Wilk test. For parametric data, One-Way ANOVA was used, followed by the Tukey's test. For non-parametric data, the Kruskal-Wallis test was used, followed by Dunn's post-test. Data are expressed as mean ± standard deviation, median, minimum, and maximum. GraphPad Prism software was used. The level of significance for consideration was $p < 0.05$.

4. Results

4.1. Transthoracic echocardiography

After 6 months of chronic exposure to glyphosate, there were no changes in the thickness and diameter of the LV during systole and diastole. The systolic and diastolic function of the LV remained unchanged. (Table 1 e Fig. 2).

Table 1

Structural and functional echocardiographic data.

Variable	C (n=14)	LC (n=14)	HC (n=14)	p value
PWT (mm)	1.14 ± 0.04	1.19 ± 0.10	1.18 ± 0.11	0.66

% ENC END	44.92 ± 7.06	43.05 ± 12.63	42.42 ± 3.18	0.82
EF %	82 ± 0.06	62 ± 0.47	80 ± 0.03	0.72
IVRT	34.00 ± 7.28	30.88 ± 6.79	30.63 ± 4.37	0.52

Data as mean ± SD. PWT: posterior wall thickness; % ENC END: percentage of endocardial shortening; EF: Ejection Fraction (%); IVRT (ms) Left ventricular isovolumetric relaxation time; C= control; LC= low concentration; HC= high concentration. Data were expressed as mean±standard deviation. One-Way Anova followed by Tukey. p value <0.05.

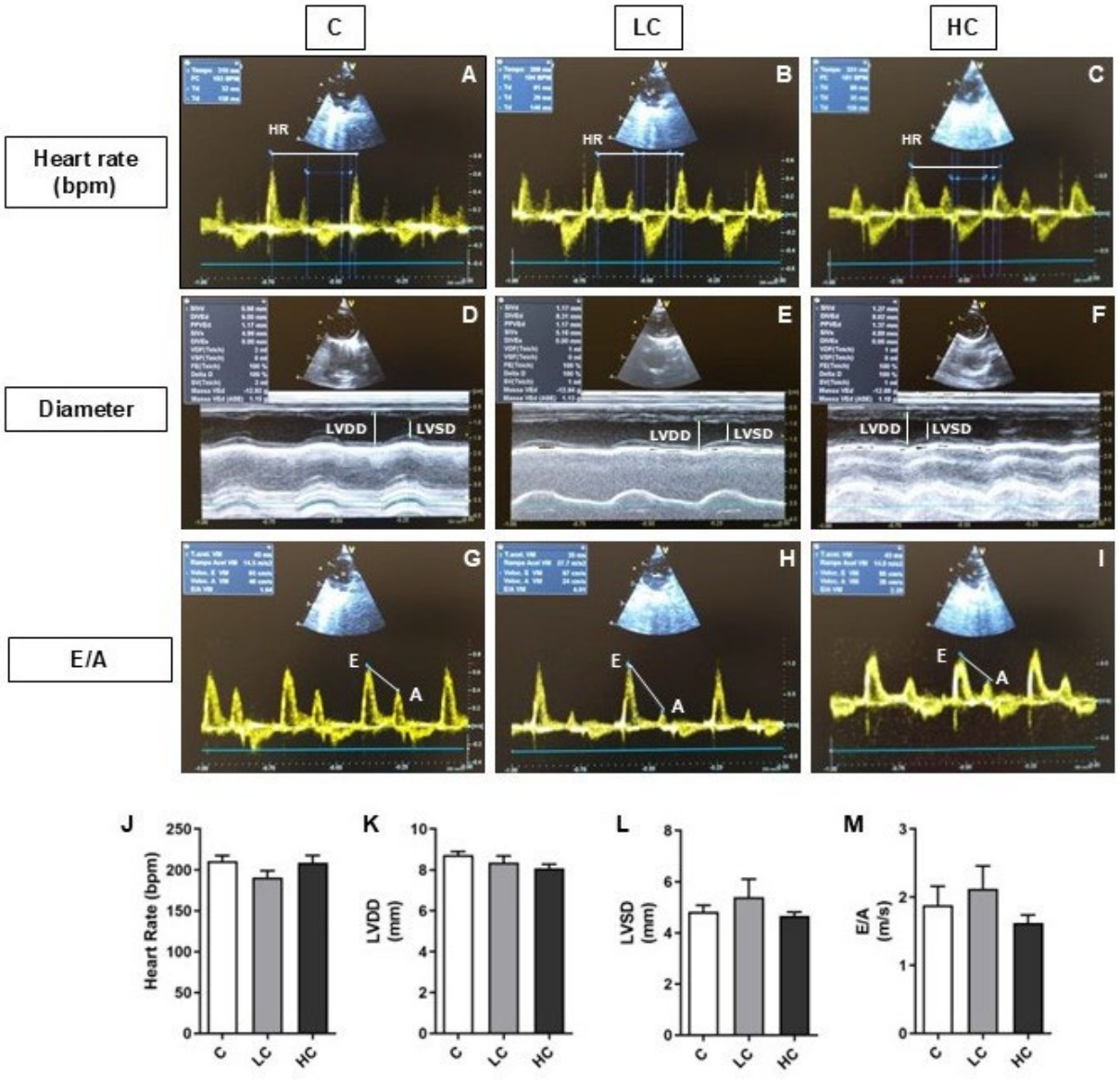

Fig. 2. Illustration of echocardiographic analysis. Heart rate in beats per minute (HR [bpm) (A, B, C and J), Left ventricular diastolic and systolic diameter (LVDD ans LVSD) (D, E, F,

K and L) and Relationship between E wave and A wave (E/A) (G, H, I and M). C=control; LC=low concentration; HC= high concentration. One-Way Anova followed by Tukey. p value <0.05.

4.2. Anatomical and histomorphometric evaluations

There were no changes in the size of the atria, right and left ventricles, and in the values normalized by final body weight (Table 2).

Table 2
Anatomical and histomorphometric data.

Variable	C (n=14)	LC (n=14)	HC (n=14)	p value
FBW (g)	444.1 ± 25.01	452.3 (383.5-471.4)	436.7 ± 34.41	0.775
ATs (g)	0.10 ± 0.01	0.09 (0.06-0.15)	0.10 ± 0.01	0.922
RV (g)	0.26 ± 0.04	0.26 (0.20-0.30)	0.25 ± 0.03	0.727
LV (g)	0.99 ± 0.05	1.00 (0.84-1.03)	0.95 ± 0.08	0.434
AT/FBW (g)	0.22 ± 0.03	0.23 (0.14-0.32)	0.24 ± 0.03	0.063
RV/FBW (g)	0.59 ± 0.10	0.60 (046-0.64)	0.57 ± 0.09	0.862
LV/FBW (g)	2.19(0.19-2.37)	2.19 (1.95-2.37)	2.18 ± 0.16	0.932

Data as mean ± SD. FBW: final body weight; ATs: atria; RV: right ventricle; LV: Left ventricle; AT/FBW: relationship between atria and final body weight; RV/FBW: relationship between right ventricle and final body weight; LV/FBW: relationship between left ventricle and final body weight; (g): grams; C = control; LC= low concentration; HC= high concentration. Anova followed by Tukey. p value <0.05.

The evaluation of cardiomyocyte area showed no changes in size (Fig. 3).

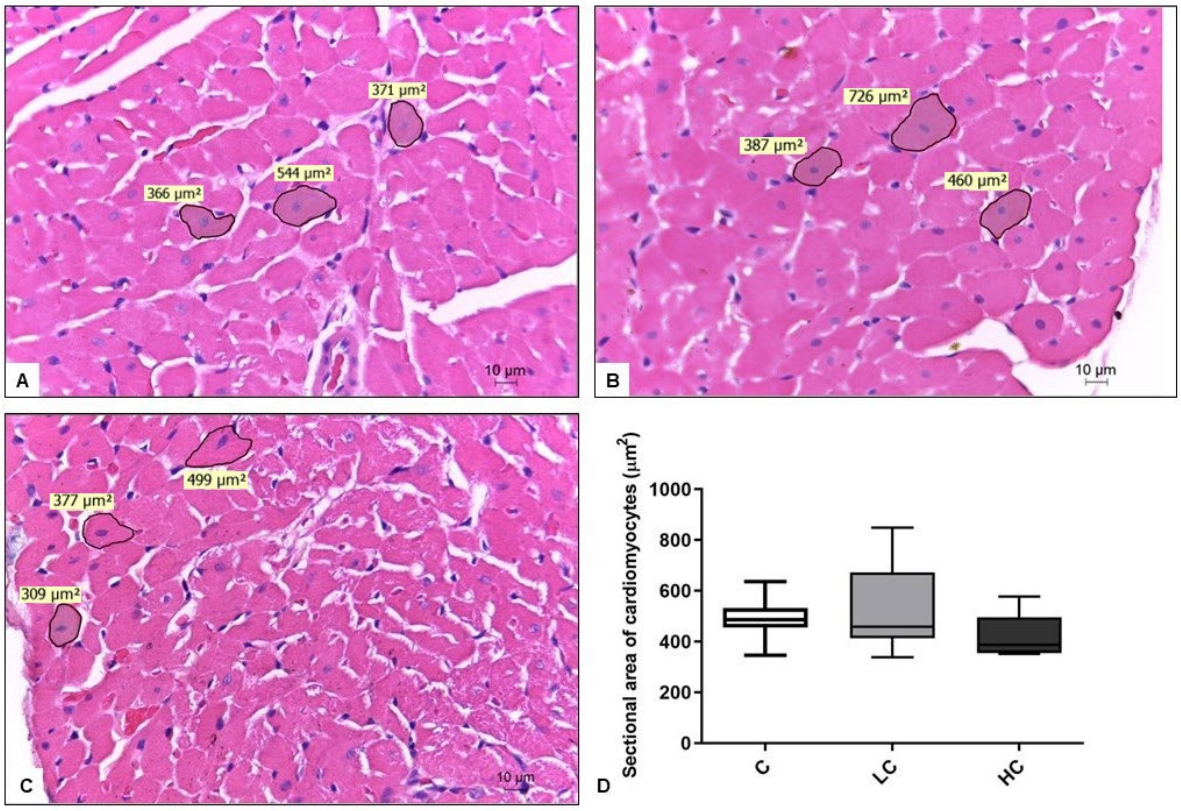

Fig. 3. Area of cardiomyocytes in the epicardial region stained in hematoxylin–eosin, 40x objective and 400x magnification. A. C = control; B. LC= low concentration; C. HC= high concentration; D. Histomorphometric analysis. Data were expressed as deviation or median (maximum and minimum). Kruskal-Wallis followed by Dunn. p value <0.05.

4.3. Fibrosis measurements

From analysis of the collected samples, it was evident that there was an increase in cardiac collagen in the HC group compared with C ($p < 0.04$). The fractal dimension and images under polarized light showed no significant differences in collagen types I and III between groups ($p>0.05$). (Fig. 4).

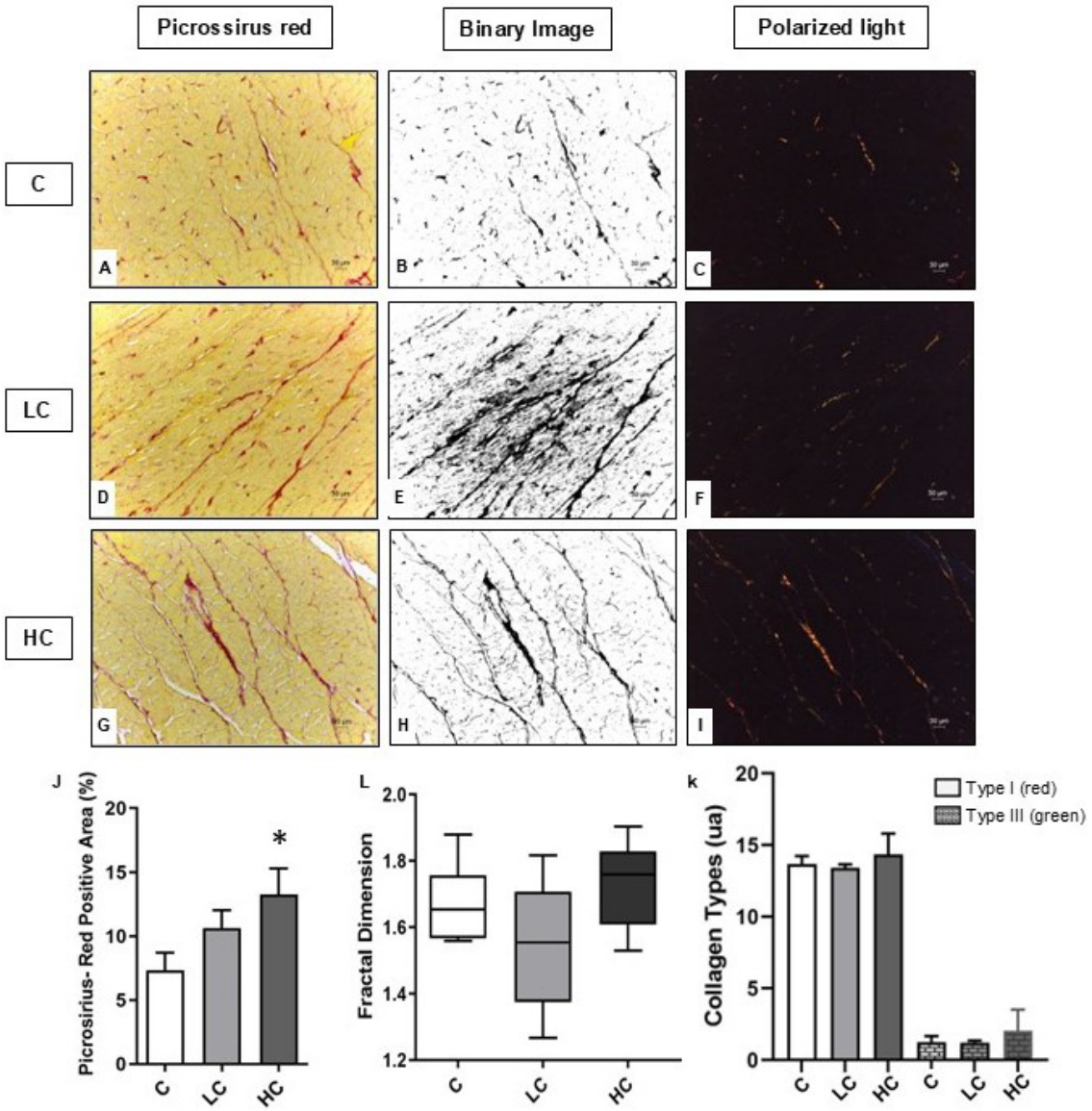

Fig. 4. Picrosirius red. Left ventricle histological (A, D, and G) sections were stained by the picrosirius red technique (PSR) and viewed with 20x objective and 200x magnification. In B, E and H correspond to the PSR images after the binarization process. Note that the collagen fibers are black and the rest of the cell (cytoplasm, plasma membrane, and other elements) is white. PSR observed under polarized (C, F and I) light with 20x objective and 200x

magnification. Glyphosate in high doses altered the area stained by PSR (J). Quantitative analysis of collagen types (K). Fractal dimension of collagen types (L). C= control; LC= low concentration; HC= high concentration. Data were expressed as mean±standard deviation or median (maximum and minimum). One-way ANOVA and Tukey's test. *p < 0.04 compared to C.

4.4. Fractal Dimension

There were no alterations in the fractal dimension between the evaluated groups. (Fig. 5).

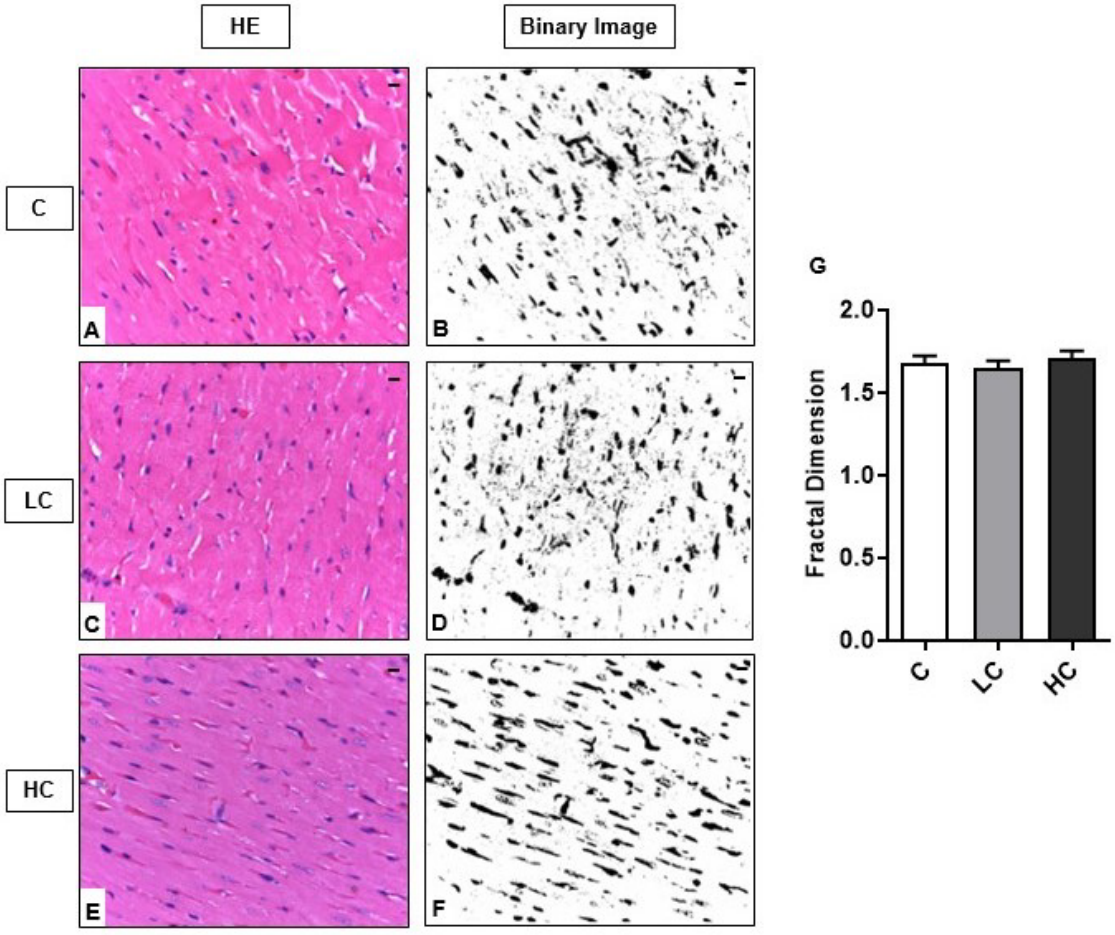

Fig. 5. Fractal Dimension**.** Left ventricle histological sections from stained with Hematoxylin and Eosin (HE) (A, C and E). B, D and F correspond to the HE images after the binarization process. Note that the nucleus of the cell is black and the rest of the cell (cytoplasm, plasma membrane, and other elements) is white. Glyphosate did not change the nuclear organization as can be seen in G. 40x objective and 400x magnification. C = control; LC= low

concentration; HC= high concentration. Data were expressed as mean±standard deviation or median (maximum and minimum). One-Way Anova followed by Tukey. p value <0.05.

4.5. Immunohistochemical analysis for the evaluation of inflammatory and apoptotic markers

After immunohistochemical analysis of the LV, we observed a decrease in TNF-α cytokine in the LC group ($p < 0.0014$) and in the HC group ($p < 0.0010$) compared to the C group (Fig.6.). There were no significant changes in the ratio Bax/Bcl-2 (Fig.7.), and protein NF-kB.

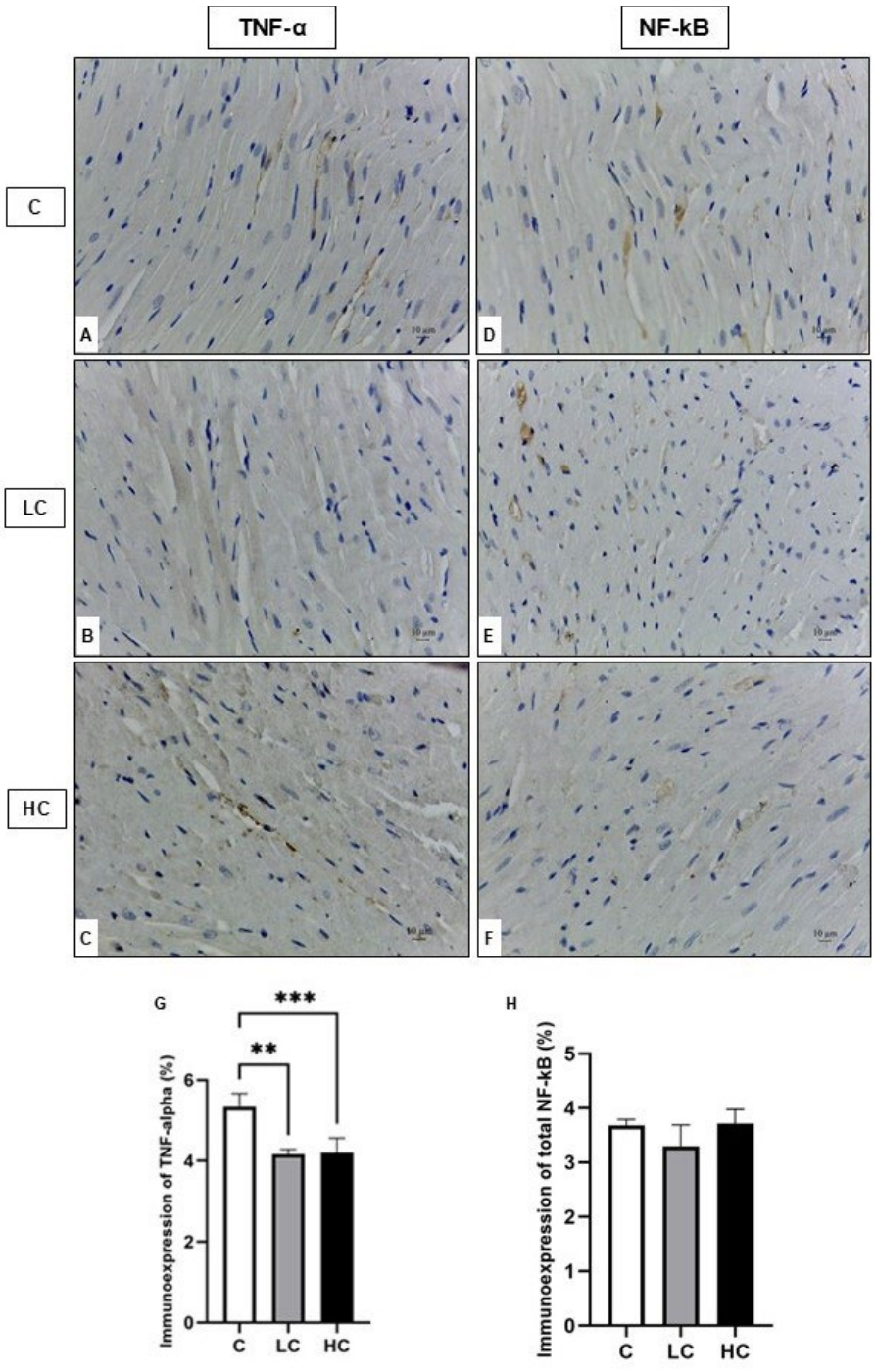

Fig. 6. Immunohistochemistry of the inflammatory pathway. A, B, C and G show total TNF-α immunoexpression in the three groups. D, E, F and H show total NF-kB immunoexpression in the three groups. 40x objective and 400x magnification. C= control; LC= low concentration; HC= high concentration. One-way ANOVA and Tukey's test. **p < 0.0014 compared to C, ***p < 0.0010 compared to C.

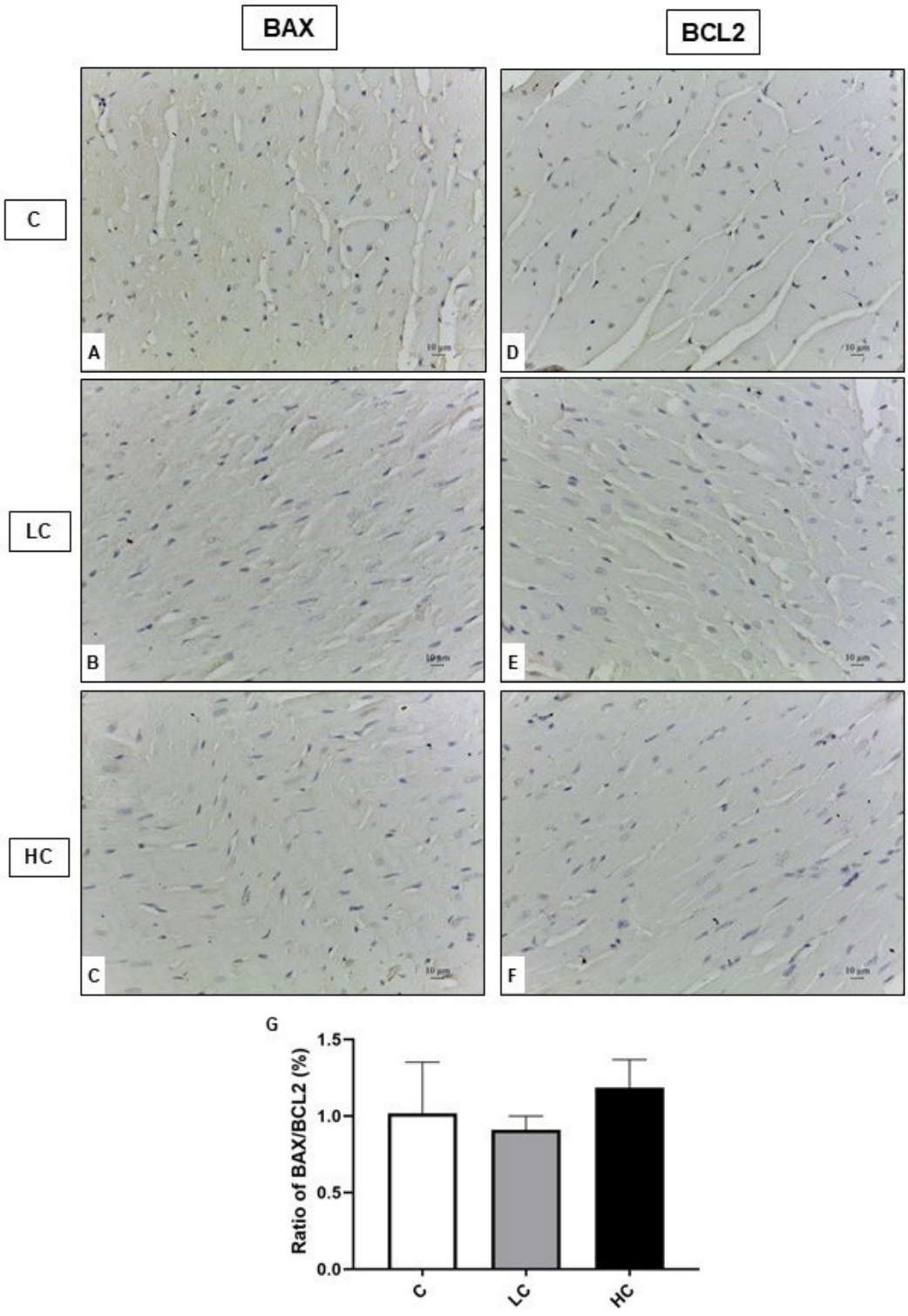

Fig. 7. Immunohistochemistry of the apoptotic pathway. A, B and C show total BAX immunoexpression in the three groups. D, E and F show total Bcl-2 immunoexpression in the three groups. G shows total BAX/BCL-2 immunoexpression. 40x objective and 400x magnification. C = control; LC= low concentration; HC= high concentration. One-way ANOVA and Tukey's test. p value <0.05.

4.6. Biomarker of oxidative stress

The different doses of inhalational exposures did not cause alteration in the levels of LPO and PCO, or in the antioxidant enzymes SOD, GSH, GT and FRAP. On the other hand, GST showed a decrease in the high concentration group compared to the control group and CAT showed an increase in the high concentration group compared to the control group (Fig.8).

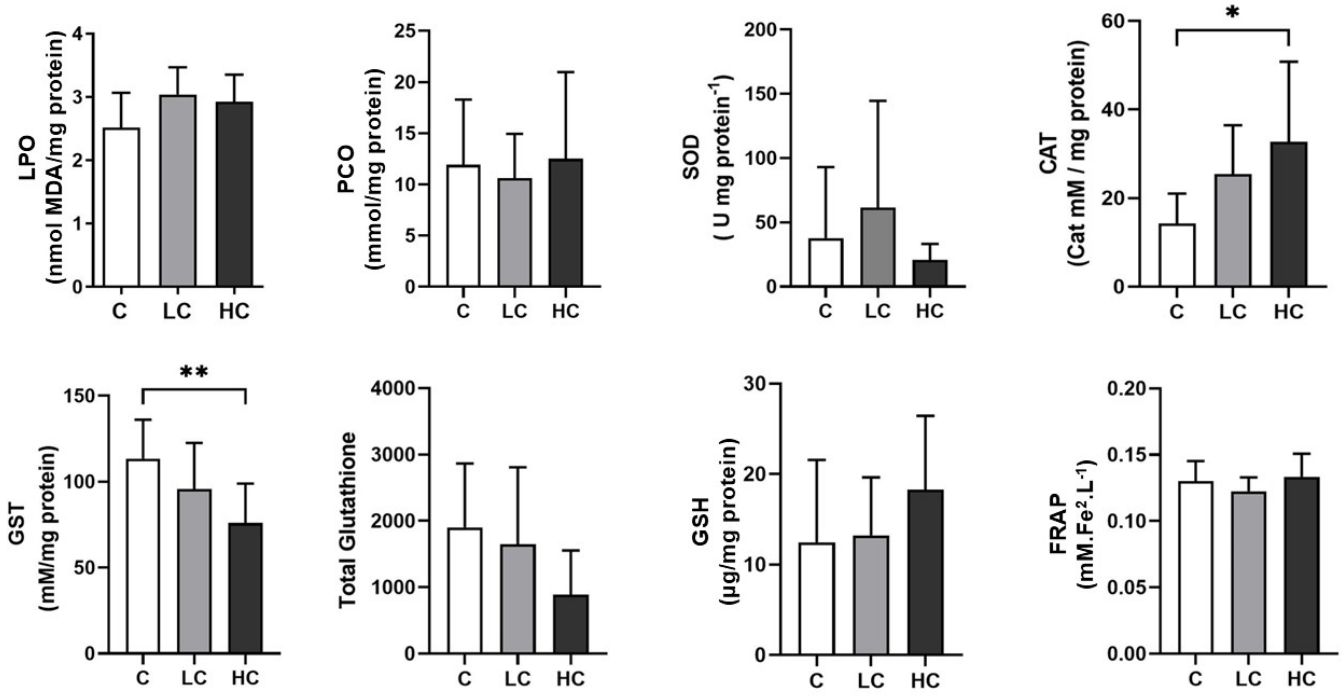

Fig. 8. Biomarker of oxidative stress. A. Lipid peroxidation (LPO). B. Protein carbonylation (PCO), C. Superoxide dismutase (SOD), D. Catalase (CAT), E. Glutathione-S-transferase (GST), F. Total glutathione (TG), G. Reduced levels of glutathione (GSH) and H Ferric reducing antioxidant power (FRAP) in cardiac cell supernatant. C = control; LC= low concentration; HC= high concentration. One-way ANOVA and Tukey's test. *p < 0.04 compared to C, **p < 0.008 compared to C.

5. Discussion

The main findings of the present study indicate that glyphosate-based herbicide, when administered by inhalation at high doses in rats over a chronic period, promoted changes in cardiac remodeling without causing functional impairment. Alterations in antioxidant enzymes, increased cardiac fibrosis, and reduced inflammatory response demonstrate adaptive responses of the heart (Fig.9.).

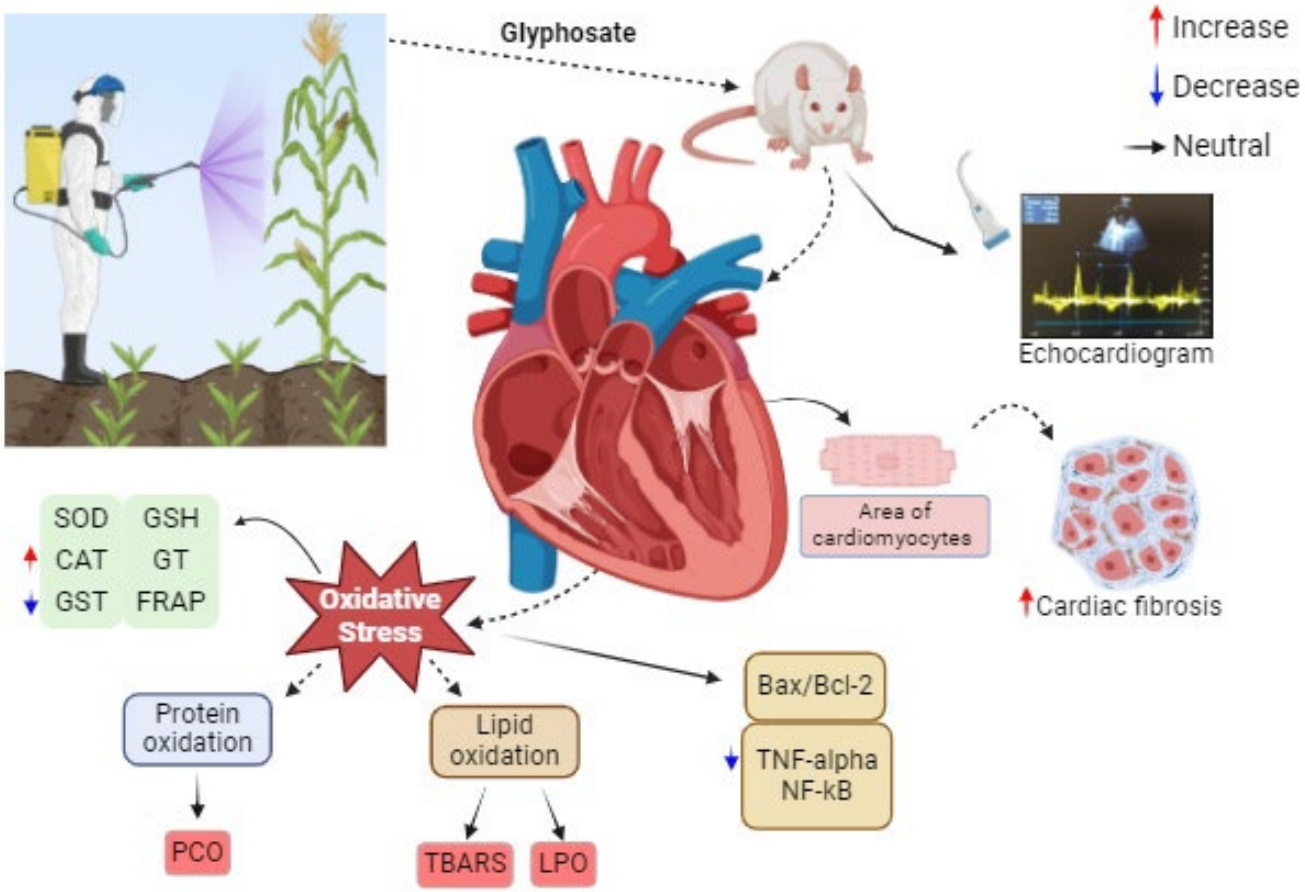

Fig. 9. Schematic figure summarizing main findings. The graphical summary was created using BioRender. Lipid peroxidation (LPO), Protein carbonylation (PCO), Superoxide dismutase (SOD), Catalase (CAT), Glutathione-S-transferase (GST), Total glutathione (TG), Reduced levels of glutathione (GSH), Ferric reducing antioxidant power (FRAP), Thiobarbituric acid (TBARS), Tumor necrosis factor alpha (TNF-alpha), Nuclear factor kappa B (NF-kB), B-cell CLL/lymphoma2 (BAX) and Bcl2-associated X protein (BCL-2).

The study results found no alterations in the systolic or diastolic function of the left ventricle (LV). A study performed with adult male wild-type (WT) and knockout mice administered an intraperitoneal injection (45 mg/kg, IP) of paraquat, a non-selective

herbicide, for 48 hours, showed a significant increase in the left ventricular end-systolic diameter (LVESD) and a decrease in fractional shortening, without affecting other geometric parameters (LV posterior wall thickness, septal thickness, LVEDD, and heart rate) (45). These alterations are related to an acute response to this type of herbicide, and in our study, the structural and functional cardiac maintenance is possibly linked to protective responses activated chronically.

No structural alterations were observed in the cardiomyocyte area or nuclear organization. Our data corroborate the study by Maia et al. (11), in which the results showed no increase in LV and RV thickness in any of the animals exposed to glyphosate via inhalation and oral administration over a subchronic period.

Our results indicated an increase in collagen in the high-dose group. Although collagen increased in our study as a possible adaptive cardiac response, there were no repercussions on LV diastolic function, indicating a compensatory cardiac response without functional impairment. Le Quilliec et al. (46) demonstrated that organochlorides and organophosphorus pesticide exposure, both acute and prolonged is associated with structural cardiac remodeling in rats inducing myocardial fibrosis and significantly increasing the risk of ventricular arrhythmias, Pandey et al. (47) studied the effects of Roundup, a popular herbicide containing glyphosate as its active ingredient, on the livers of rats exposed over a subacute period. The results showed mild to severe hepatic fibrosis, characterized by collagen accumulation, in the group of rats orally exposed to doses of 5, 10, 25, 50, 100, and 250 mg/kg body weight (bw)/d of glyphosate for 14 days. Studies on the liver are important as the liver is a vital organ involved in xenobiotic metabolism and biotransformation (48). These studies suggest that toxicity appears to be herbicide-specific and varies depending on the affected organ. However, a study using glyphosate (11) over a subchronic period, with the same dose used in the current study, did not show an increase in cardiac fibrosis. Most heart diseases involve pathological myocardial remodeling, characterized by excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs), reducing tissue compliance and accelerating the progression to heart failure (49,50). Fibrotic processes are demonstrated in chronic phases where the acute inflammatory process triggers a response from fibroblasts to increase collagen production (51). Regarding collagen types, no nosso estudo there was no increase in type I and type III collagen, which might be related to the increase in other types of collagen in cardiac tissue, as is common in myocardial infarction (52). The different alterations in cardiac collagen do not always lead to functional changes, as the cardiovascular system has a high adaptive capacity (53)

Regarding the fractal dimension, there was no alteration in the cellular organization of cardiomyocytes. No studies in the literature have evaluated glyphosate-based herbicides using fractal dimension analysis. In a previous study by our group, rats were chronically exposed orally to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), which also showed no changes in cellular organization (18). This methodology allows for more precise quantification of tissue and cellular disorganization. In the field of cardiology, fractal dimension analysis has been used to quantify right ventricular structures in a rat model of pulmonary arterial hypertension and to assess myocyte preservation in isolated rat hearts, which resulted in higher fractal dimension values in the treated group (32).

A decrease was found in TNF-α cytokine expression in the high-concentration groups without changes in the NF-kB marker. This alteration in TNF- α indicates a dysfunction in the cardiac immune system. Other studies involving herbicides have also demonstrated an impact on the immune system. Two studies using atrazine (ATR), a broad-spectrum herbicide used to eliminate weeds, aimed to study the effects of prolonged ATR exposure on immune system function in rats. In one study, Sprague-Dawley rats were orally administered 0, 0.4 μmol/L, 2 μmol/L, and 10 μmol/L of ATR in water ad libitum for 24 consecutive weeks to simulate ATR contamination. The results showed that both TNF-α and interleukin-12 levels significantly decreased in the serum of ATR-treated rats, indicating that ATR suppressed the immune response (54). The other study investigated the effects of ATR on the immune system of four-week-old female C57BL/6 mice, treated orally with 0, 5, 25, or 125 mg/kg of ATR for 28 days, and the results demonstrated inhibition of cellular immune function (55).

After acute cardiac injury, there is an increase in the expression of pro-inflammatory cytokines and pro-fibrotic factors in cardiac fibroblasts, which proliferate and transform into myofibroblasts. These cells secrete high levels of collagen and other extracellular matrix proteins to maintain the structural integrity and pressure capacity of the heart, preventing myocardial dysfunction or rupture (56,57). Other studies have shown an increase in pro-inflammatory cytokines in liver, spleen of tilapia, hepatotoxicity and neuroinflammation in mice following acute and chronic exposure to the herbicide glyphosate (13,16,58).

We did not observe a change in the Bax/Bcl-2 ratio. However, Hao et al. (59) studied the effect of Roundup glyphosate at concentrations of 50 to 125 mg/mL on mitochondria-associated apoptosis and DNA damage in human alveolar carcinoma cells (A549 cells). The results showed mitochondrial membrane collapse due to the increased Bax/Bcl-2 ratio.

The BAX/BCL-2 ratio can act as a rheostat, determining cellular vulnerability to apoptosis (60). An increased ratio correlates with dysfunction and even tissue injury, as

previously reported by Asmarinah et al. Bcl-2-associated X protein (BAX) is a pro-apoptotic gene, while B-cell CLL/lymphoma 2 (BCL-2) is an anti-apoptotic gene (61). The protein balance between Bax and Bcl-2 is crucial because an excess of Bax leads to apoptosis, while a predominance of Bcl-2 inhibits this process, allowing cells to survive (62). These findings underscore the importance of understanding the molecular mechanisms of pesticide toxicity, particularly regarding their impact on apoptotic pathways, which could have broad implications for human health and disease prevention.

In the analysis of oxidative stress biomarkers, results indicated an increase in the CAT enzyme and a decrease in GST, suggesting that the heart was actively responding to the damage caused by glyphosate-based herbicides. Additionally, there were no significant changes in the biomarkers SOD, GT, GSH, and FRAP.

In the current study, we did not observe changes in lipid and protein peroxidation levels among the exposed groups. However, this does not imply that oxidative stress was absent due to glyphosate exposure, as the high-dose group showed increased activity of the antioxidant enzyme CAT. This alteration in the antioxidant profile suggests a compensatory mechanism in response to oxidative stress in these cells. A study conducted on hybrid piglets fed 0, 10, 20, or 40 mg/kg of glyphosate for 35 days showed an increase in CAT and SOD enzymes in the duodenum and elevated MDA levels as the glyphosate dose increased (63). Rieg et al. (64) demonstrated that oral exposure to glyphosate (70 mg/kg) from the 5th day of gestation and continuously until the 15th day of lactation caused an increase in LPO and PCO levels in the liver of immature rats, indicating glyphosate-induced oxidative damage to lipids and proteins. Modesto and Martinez (65) and Lajmanovich et al. (66) reported that acute glyphosate exposure could increase LPO in tadpoles and fish.

Another enzyme, GST, also recognized as an important catalyst in the biotransformation of xenobiotics, environmental pollutants, and oxidative stress byproducts (67,68), was found to be decreased in the high-dose exposed group, partially reducing the antioxidant capacity in cardiac tissue and potentially indicating insufficient detoxification of the herbicide. This result is consistent with another study where decreased GST activity was observed in tadpoles exposed for 48 hours to a commercial glyphosate formulation at concentrations of 1.85, 3.75, 7.5, 15, 30, 60, 120, and 240 mg ae/L (66).

Lipid peroxidation indicates oxidative damage to lipids, while protein carbonylation indicates oxidative damage to proteins, both resulting from increased reactive oxygen species (69). There was no significant difference in LPO and PCO levels in our study. Our data corroborate a study by Lushchak et al. (70), which used a similar method to quantify lipid

peroxidation and found that the herbicide Roundup Original also does not affect lipid peroxidation in the liver of goldfish after 96 hours of exposure. In contrast, Rieg et al.(64) demonstrated that perinatal exposure to glyphosate increases LPO and PCO levels in the liver of immature rats, indicating pesticide-induced oxidative damage to lipids and proteins. Modesto and Martinez (65) and Lajmanovich et al. (66)also reported that acute exposure to glyphosate can increase LPO in tadpoles and fish.

From a public health perspective, these findings suggest that occupational exposure to glyphosate may pose a significant risk to cardiovascular health. Safety control measures are critically important when monitoring the use of pesticides such as glyphosate. Many pesticides have been shown to cause cardiac damage. We did not assess other potential markers related to apoptotic and inflammatory pathways and their electrophysiological effects. These results underscore the need for future research to explore the molecular mechanisms involved and the potential long-term impacts on different species.

6. Conclusion

Chronic inhalation exposure to the herbicide glyphosate resulted in cardiac remodeling characterized by increased collagen with a protective adaptive response, without functional impairment.

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ANEXO 1- APROVAÇÃO ÉTICA

- Unoeste

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PEIC - Programa Especial de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"ANÁLISE MORFOFUNCIONAL CARDÍACA EM RATOS WISTAR SUBMETIDOS A EXPOSIÇÃO CRÔNICA AO HERBICIDA GLIFOSATO"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **7093** e tendo como participante(s) **RAISSA DE OLIVEIRA MANTOVANI (discente), BIANCA APARECIDA CAMPOS COGO (discente), GLAURA SCANTAMBURLO ALVES FERNANDES (participante externo/voluntário), RENATA CALCIOLARI ROSSI (docente), RENATA MANO SCATAMBURLO BIFARONI (docente), FRANCIS LOPES PACAGNELLI (orientador responsável)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 07/12/2021.

MATERIAL ARMAZENADO/DOADO

Protocolo(s)	Data Aprovação	Armazenado (local)	É doação	Detalhes armazenamento
5684	13/11/2019	UNOESTE	SIM	Laboratório de genética

Presidente Prudente, 3 de Outubro de 2022.

Prof. Dr. Jair Rodrigues Garcia Jr.
Docente Responsável pela CPDI

https://www.unoeste.br/SGP/certificados/ver.asp?h=ff160ef5f9deefcc60e124408cd47442

Prof. Dr. Felipe Rydygier de Ruediger
Coordenador da CEUA - UNOESTE

ANEXO 2- NORMAS DE PUBLICAÇÃO DA REVISTA LIFE SCIENCES

Organization of the manuscript

Beginning with the first page, present your manuscript in the order below:
1. Title: First letter capitalized, subsequent letters in lower case. Avoid abbreviations.
2a. Names of all authors.
2b. Affiliations of all authors. If necessary, use superscripted lowercase letters after the author's name to distinguish affiliations.
3. Author to whom proofs and correspondence should be sent, including name, mailing address, telephone and fax numbers, and e-mail address.
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Aims:
Main methods:
Key findings:
Significance:
5. Key words for indexing purposes (a maximum of six can be entered). In addition to key words from the title, please suggest other terms that help define the study. We encourage authors to test the relevance of their key words by using them for a database search and comparing the results with the topic of their own paper.

Word limits: In **full papers**, individual sections should be no longer than Abstract 250 words, Introduction 500 words, Discussion 1500 words, Conclusion 150 words. Materials and Methods and Results sections should be concise but there is no formal word limit.

Headings: Papers must include the major headings Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgments, and References. Include subheadings as appropriate. Review articles must contain Abstract and Introduction, with subsequent headings and subheadings as appropriate.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

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Results should be clear and concise.

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Conclusions

Present the conclusions of the study in a short Conclusions section.

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Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

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Abbreviations used as names of cell lines do not need to be explained, but the species and tissue of origin should be made clear in text the first time the cell line is mentioned. Examples: "the human colonic adenocarcinoma cell line Caco-2" or "the porcine renal endothelial cell line LLC-PK1".

Western blots images

Life Sciences requires submission of the whole uncropped images of the original western blots in triplicate that contributed to the quantitative analysis, from which figures have been derived. Please submit as Supplementary Figure(s). **Please note that this is mandatory when western blots are shown**. Please see Example of original western blot for three repeats

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or

otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Please note that funding information must appear under the Acknowledgments heading.

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List funding sources in this standard way to facilitate compliance to funder's requirements:

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List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

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Reference to a journal publication with an article number: [2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book: [3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book: [4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website: [5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

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2 ARTIGO CIENTÍFICO 2

TRANSLATION, CULTURAL ADAPTATION AND AGREEMENT ASSESSMENT OF THE SYRCLE-ROB TOOL INTO BRAZILIAN-PORTUGUESE

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ABSTRACT

Introduction: Systematic reviews and meta-analyses (RSMA) of preclinical studies have been used to increase the translational validity of research and to accelerate the transposition of basic to applied science. As with clinical RSMA, it is necessary to assess the risk of bias in studies, as low quality or bias can invalidate the results. In pre-clinical studies, the most used instrument is the SYRCLE-RoB, adapted from the Cochrane risk of bias tool and composed of 10 items. The translation, validation and analysis of this tool is important, especially so that it can be more widely disseminated and applied in RSMAs in the area of pre-clinical studies. **Objective**: Translate, adapt and validate the agreement of the SYRCLE-RoB tool into Brazilian Portuguese. **Methods**: The translation of the tool was carried out following an international guide. After translation, back-translation and approval of the version by the creators, the tool, in Portuguese, was evaluated for agreement. For this, eleven articles involving experimental animals, which had intervention and in English were randomly chosen after consulting PubMed. The translated and adapted version of SYRCLE-RoB was applied in each study by 2 researchers. Statistical analysis: To analyze agreement between evaluators, the Fleiss kappa index was calculated for each item. Results: Considering that SYRCLE-RoB contains 10 analysis items and 11 articles were used to evaluate, we calculated the percentage of agreement in 111 conditions. Total agreement was 53.33% (Cohen's Kappa= 0.18) considering all items in the tool. The average agreement rate for items 1-7 was 76.19% (Cohen's Kappa = 0.47). **Conclusion**: The SYRCLE-RoB instrument translated into Brazilian Portuguese showed moderate reliability, which can be considered valid for application in Portuguese as a form of methodological evaluation in pre-clinical studies.

Keywords: Risk of bias, Animal studies, Systematic reviews, Tool, Meta-analyses

INTRODUCTION

Many studies in Brazil and worldwide have been carried out using animal experimentation models. Research on animals has grown, leading to an overuse of animals for experimentation. This also led to problems of replicability and external validity in pre-clinical research. Preclinical systematic reviews emerged, as a tool to comprehensively analyze scientific publication in experimental animals' studies (Hooijmans et al. 2012).

One of the key features of systematic reviews is the analysis of risk of bias (Macleod et al. 2009). The assessment of risk of bias in human clinical trials is performed using instruments such as the Cochrane Collaboration Risk of Bias (RoB) Tool version 2.0 (Higgins et al. 2011, Sterne et al. 2019). An adaptation of this tool, based on the its original version, was developed by the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE – Nijmegen, the Netherlands), to assess the risks of scientific methodological bias in animal experimentation (Hooijmans et al. 2014). SYRCLE RoB tool was developed to analyze the risk of scientific bias in experimental animal research, encompassing the following types of bias: selection bias (sequence generation, basal characteristics, and allocation concealment), execution bias (random housing and blinding), detection bias (random assessment of outcomes and blinding), attrition bias (incomplete outcomes), reporting bias (selective outcome reporting), and others (other sources of bias). The questionnaire is intended for qualitative appraisal of data and each item is designed to be answered with the responses "yes" (for low risk of bias), "no" (for high risk of bias), or "uncertain" (for when the risk of bias cannot be determined) (Hooijmans et al. 2014).

This tool was initially developed in English and no translation or cultural adaptation has been developed to this date. Although most of the literature is currently available in English, many systematic reviews are performed by researchers whose English is not the primary language. Therefore, having a tool translated into other languages might increasing the precision and performance of risk of bias analyses by providing instructions adapted in other languages. Also, considering the growing number of systematic review studies in animal models being performed, the translation of this instrument can disseminate and encourage researchers to carry

out systematic reviews of preclinical studies. Finally, translating it into Brazilian Portuguese seems like a relevant achievement, considering the relevance of Brazilian output in the field of preclinical systematic reviews. According to a recent meta-epidemiological study, Brazil was ranked seconds on a list of countries sorted by their publication record, accounting for 11.7% of all preclinical systematic reviews published up to 2019 (Hunniford et al. 2021). Therefore, this study aimed to translate the SYRCLE RoB tool into Brazilian Portuguese.

METHODS

Study design

The study was divided into two phases. Phase 1 consisted of translation, cross-cultural adaptation, and content validity assessment by 25 researchers. Phase 2 included reliability evaluation through the application by two evaluators of the translated tool in pre-clinical articles.

First, permission to translate and cross-culturally adapt the Risk of Bias (RoB) tool to Brasilian Portuguese language was obtained from the authors of the SYRCLE RoB tool original version. (Hooijmans et al. 2014). The study phases are summarized in Figure 1.

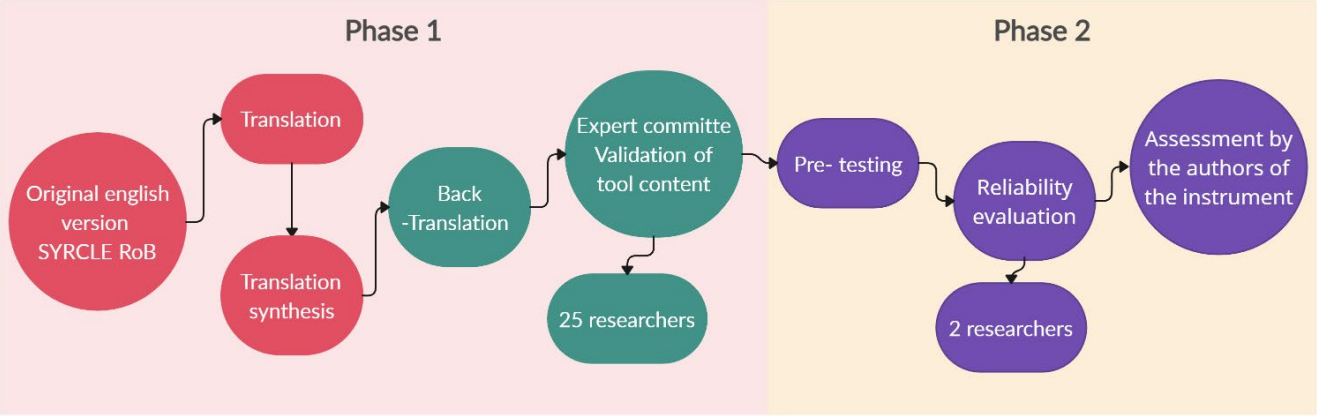

Fig. 1. Flowchart of the translation, cross-cultural adaptation and validity assessment stages of the SYRCLE RoB tool.

Translation, cross-cultural adaptation and validity assessment

The translation of the tool was conducted in 2021 and 2022. Translation and validation were carried out based on protocols validated by Guillemin et al. 1993, Xie et al. 2018, and Santos et al. 2019, and adapted in five steps.

- Step 1 – In the initial translation phase, two bilingual Brazilian researchers with experience in the SYRCLE RoB tool (GNP and AGB) independently translated the scale into Brazilian Portuguese.
- Step 2 – In the translation synthesis phase, the translators and two other researchers (TBM and APCFF) prepared a single consensus version of the translated scale.
- Step 3 – In the English back-translation phase, two native English speakers (British, RH and American, ES), who had no previous contact with the original version of the scale, independently translated the consensually approved version in Brazilian Portuguese to English.
- Step 4 – In the consensus version and evaluation phase, the tool re-translated into English was submitted for evaluation by the team that developed the tool (CH and MRH). After this analysis, the tool needed adjustments, which involved a Brazilian researcher (GNP) with experience in systematic reviews in animal models and in the SYRCLE RoB tool, and two bilingual researchers (FLP and APCFF). The version was again translated into Portuguese, back-translated into English, and again sent to the development team for approval.
- Step 5 – Pre-test of the final version: 25 bilingual researchers working with experimental animal models in rats in several fields evaluated the tool translated in relation to the level of understanding of the items (possible answers: fully understand, partially understand, and do not understand).

Reliability evaluation

The reliability evaluation was made based on a random list of articles disclosing studies with preclinical animals. The list was based on a PubMed search performed on August 26th, 2021, and limited to studies published in 2021, resulting in 7948 search records. After this selection, a list of 30 articles were randomized (using the randomizer.org tool), which were evaluated according to the following exclusion criteria: 1. Non-original articles (ex.: reviews and letters to the editor); 2. Studies performed in humans beings; 3. Veterinary studies; 4. Studies performed with free-

living animals; 5. Studies with no interventions, and 6. studies for which a full-text in English could not be found. A sample of 11 articles remained after the eligibility analysis.

Each study was evaluated using the Brazilian Portuguese translated version of the SYRCLE RoB tool by 2 independent researchers (HBN and GAN) with previous experience in working with animal models, but without previous experience in the SYRCLE RoB tool. This intended to evaluate whether simply the tool itself and the supporting material (including signaling questions) was sufficient for a reliable risk of bias assessment. Following, the same reviewers had a meeting with a researcher with experience in using the original SYRCLE RoB tool (GNP), in order to provide training and clarifying practical points on the use of the tool. Following, the evaluation was repeated to check the relevance of previous training about the tool for a proper risk of bias assessment.

Agreement rates

The agreement between both reviewers, considering the 11 articles evaluated and the 11 items comprising the SYRCLE RoB tool was calculated, both before and after the training session. Agreement rates were calculated as percentages, as well as using the Cohen's Kappa index. The value of the Kappa agreement coefficient can vary from -1 to +1, where a negative value indicates that the agreement between the evaluators was lower than the agreement expected by chance. With -1 we indicate that there was no agreement, 0 indicates that agreement is no better than chance, and values greater than 0 represent increasing agreement for the evaluators, up to a maximum value of + 1, indicating perfect agreement (Landis and Koch, 1977).

RESULTS

Brazilian Portuguese version of the SYRCLE RoB tool

The translated version of SYRCLE RoB tool into Brazilian Portuguese is described in Table 1 and the supporting material with signaling questions for each item is disclosed in Table 2.

Table 1 – Tool for risk of bias assessment.

Item	Tipo de viés	Domínio	Descrição do domínio	Julgamento pelos autores
1	Viés de seleção	Geração de sequência	Descrever os métodos usados, se houver, para gerar a sequência de alocação em detalhes suficientes para permitir avaliar se ela pode produzir grupos comparáveis.	A sequência de alocação foi gerada e aplicada adequadamente? (*)
2	Viés de seleção	Características basais	Descrever todos os fatores prognósticos ou características dos animais (se houver) que são comparados a fim de julgar se os grupos intervenção e controle eram similares no começo do experimento.	Os grupos eram similares no momento basal ou foram ajustados para confundidores nas análises.
3	Viés de performance	Ocultação da alocação	Descrever o método usado para ocultar a sequência de alocação com detalhes suficientes para determinar se as alocações de intervenção poderiam ter sido previstas antes ou durante a inclusão nos diferentes grupos.	A alocação foi adequadamente ocultada? (*)
4	Viés de performance	Alojamento aleatório	Descrever todas as medidas usadas, se houver, para alojar os animais aleatoriamente na sala.	Os animais foram alojados de forma aleatória durante o experimento?
5	Viés de execução	Cegamento	Descrever todas as medidas, se houver, para cegar cuidadores e pesquisadores de saber qual intervenção cada animal recebeu. Fornecer todas as informações sobre se o cegamento foi efetivo.	Os cuidados e/ou pesquisadores estavam cegados sobre qual intervenção cada animal recebeu durante o experimento?
6	Viés de detecção	Avaliação aleatória dos desfechos	Descrever se os animais foram ou não selecionados aleatoriamente para avaliação dos desfechos e quais métodos para selecionar os animais, se houver, foram utilizados.	Os animais foram selecionados aleatoriamente para avaliação dos desfechos?

7	Viés de detecção	Cegamento	Descrever todas as medidas usadas, se houver, para cegar os avaliadores dos desfechos de saber qual intervenção cada animal recebeu. Fornece informações sobre se o cegamento foi efetivo.	Os avaliadores dos desfechos foram cegados?
8	Viés de atrito	Desfechos incompletos	Descrever se os dados relacionados a cada desfecho principal estão completos, incluindo perdas e exclusões nas análises. Declarar se perdas e exclusões foram relatadas, o número em cada grupo (comparado com o total de animais aleatorizados), razões para perdas e exclusões e quaisquer reinclusões nas análises.	Desfechos com dados incompletos foram adequadamente abordados? (*)
9	Viés de relato	Relato de desfecho seletivo	Indicar como o relato seletivo de desfecho foi examinado e o que foi encontrado.	O estudo está livre de relato seletivo de desfecho? (*)
10	Outro	Outras fontes de viés	Declarar quaisquer preocupações importantes sobre vieses não cobertos por outros domínios desta ferramenta.	O estudo aparentemente está livre de outros problemas que poderiam resultar em um alto risco de viés? (*)

(*): Itens de acordo com os itens da ferramenta Cochrane Risk of Bias.

Table 2 – Perguntas Guia

As perguntas-guia (PG) adicionais são incluídas para auxiliar a avaliação. “Sim” indica baixo 0risco de viés; Não” indica alto risco de viés; “Incerto” indica risco de viés incerto. Se uma das perguntas é respondida com “Não”, isto indica alto risco de viés para aquele tópico específico.

1. A sequência de alocação foi gerada e aplicada adequadamente?
    - PG 1.1: Os pesquisadores descreveram um componente aleatório no processo de geração de sequência e alocação dos animais em diferentes grupos? (Sim / Não / Incerto)
        - Referência a uma tabela de números aleatórios.
        - Uso de um gerador computacional de números aleatórios.
    - Informações adicionais:
        - Exemplos de abordagem não aleatória.
            - Alocação por julgamento ou preferência do pesquisador.
            - Alocação baseada nos resultados de testes de laboratório ou de uma série de testes.
            - Alocação por disponibilidade de intervenção.
            - Sequência gerada por data de nascimento par ou ímpar.
            - Sequência gerada por alguma regra baseada no número do animal ou da caixa.

2. Os grupos eram similares no momento basal ou foram ajustados para confundidores nas análises?
    - PG 2.1: A distribuição das características relevantes foi balanceada para os grupos intervenção e controle? (Sim / Não / Incerto).
    - PG 2.2: Se relevante, os investigadores ajustaram adequadamente as análises para distribuição desigual de alguma característica basal importante? (Sim / Não / Incerto).
    - PG 2.3: O momento de indução da doença foi adequado? (Sim / Não / Incerto).
    - Informações adicionais
        - O número e tipo das características bases são dependentes da pergunta da revisão. Portanto, antes de iniciar a avaliação de risco de viés os revisores devem discutir quais características basais precisam ser comparáveis entre os grupos. Por exemplo, em um RS que investiga os efeitos da hipotermia no tamanho do infarto, deveriam ser similares entre os grupos no início do estudo as seguintes variáveis: distribuição de gênero, peso do ventrículo esquerdo, frequência cardíaca e pressão arterial.
        - A descrição das características basais e/ou confundidores geralmente contém:
            - O sexo, idade e peso dos animais.
            - Valores basais dos desfechos de interesse do estudo.

- Momento de indução da doença: Em alguns estudos de prevenção, a doença é induzida depois da alocação da intervenção. Por exemplo, em um experimento com suplementação preventiva de probioóticos em pancreatite aguda, a pancreatite é induzida depois da alocação dos animais para o grupo que receberá probiótico ou para o grupo controle. Para reduzir desequilíbrios em medidas basais, o momento da indução da doença deve ser igual para ambos os grupos de tratamento. Exemplos de momento de indução de doença adequados:
  - A doença foi induzida antes da aleatorização da intervenção.
  - A doença foi induzida depois da aleatorização da intervenção, mas o momento da indução da doença foi aleatorizado, e a pessoa responsável pela indução da doença foi adequadamente cegado sobre qual intervenção cada animal receberia.

3. A alocação em diferentes grupos foi adequadamente ocultada durante o experimento?
   - PG 3.1: O pesquisador responsável por alocar os animais ao grupo intervenção ou ao grupo controle não poderia prever a atribuição aos grupos devido a um dos seguintes métodos ou a métodos equivalentes? (Sim / Não / Incerto)
     - Codificação da alocação em grupo experimental e grupo controle por uma terceira parte.
     - Aleatorização realizada por uma terceira parte.
     - Uso de envelopes opacos, selados, numerados sequencialmente.
   - Informações adicionais:
     - Exemplos em que os pesquisadores possivelmente poderiam prever a atribuição aos grupos:
       - Cronograma de aleatorização aberto.
       - Envelopes sem proteção adequada.
       - Alternância ou rotação.
       - Alocação baseada na data de nascimento.
       - Alocação baseada no número do animal.
       - Qualquer outro procedimento explicitamente não ocultado de abordagem não aleatória.

4. Os animais foram alojados aleatoriamente durante o experimento?
   - PG 4.1: Os pesquisadores dispuseram as caixas, gaiolas ou animais de maneira aleatória no local onde os animais são mantidos (biotério/sala de animais/vivário)? (Sim / Não / Incerto).
     - Animais foram selecionados aleatoriamente durante a avaliação do desfecho (usar perguntas-guia do item 6).

- PG 4.2: É improvável que o desfecho ou a avaliação do desfecho tenha sido influenciada pelo alojamento não aleatório dos animais? (Sim / Não / Incerto).
    - Os animais de diferentes grupos experimentais vivem juntos em uma mesma gaiola/pasto (ex.: condições de alojamento são idênticas).
- Informações adicionais.
    - Exemplos de pesquisadores que usam abordagem não aleatório para dispor as gaiolas/caixas.
        - Grupos experimentais foram estudados em locais diferentes (ex.: grupo A no laboratório A ou na prateleira A; grupo B no laboratório B ou na prateleira B).

5. Os cuidadores e/ou investigadores estavam cegados sobre qual intervenção cada animal recebeu durante o experimento?
    - PG 5.1: O cegamento dos cuidadores e investigadores foi assegurado e foi improvável que esse cegamento tenha sido quebrado? (Sim / Não / Incerto).
        - Os cartões de identificação dos animais e/ou das gaiolas foram codificados e têm aparência idênticas.
        - Frascos de drogas/medicamentos identificados sequencialmente são têm aparência idêntica.
        - As circunstâncias durante a intervenção são especificadas e semelhantes em ambos os grupos.
        - As condições de alojamento dos animais durante o experimento são aleatorizadas dentro da sala (use os critérios do item 4).
    - Informações adicionais
        - Exemplos de cegamento inapropriados
            - Etiquetas ou rótulos coloridos nas gaiolas (ex..: vermelho para o grupo A, amarelo para o grupo B).
            - As condições de alojamento dos animais não são randomizadas na sala durante o experimento (use os critérios do item 4).
            - Diferenças esperadas em efeitos visíveis entre grupos experimental e controle.
            - O indivíduo que prepara o experimento é o mesmo que conduz e analisa o experimento.
            - Circunstâncias durante a intervenção não são semelhantes em ambos os grupos.
        - Exemplos em que circunstâncias durante a intervenção não foram semelhantes:
            - Momento da administração do placebo e da droga experimental foi diferente.
            - Instrumentos usados para realizar os experimentos diferem entre os grupos experimental e controle (ex..: experimentos sobre efeitos da pressão abdominal, em

que grupo experimental recebe cirurgia e agulha para aumentar pressão, enquanto o grupo controle recebe apenas cirurgia).
        - **A relevância dos itens mencionados acima depende dos experimentos. Os revisores precisam julgar quais dos itens acima mencionados poderiam causar viés nos resultados quando desiguais. Esses devem ser avaliados.

6. Os animais foram selecionados aleatoriamente para avaliação dos desfechos?
    - PG 6.1: Os investigadores selecionaram aleatoriamente um animal durante avaliação do desfecho, ou usaram um componente aleatorizado na geração de sequência para avaliação do desfecho? (Sim / Não / Incerto).
        - Exemplos de geração de sequência aleatória para avaliação de desfecho:
            - Referência a tabela de números aleatórios.
            - Uso de um programa de computador para gerar números aleatórios.
7. O avaliador de desfechos foi cegado?
    - PG 7.1: O cegamento do avaliador dos desfechos foi garantido e era improvável que o cegamento pudesse ter sido quebrado? (Sim / Não / Incerto)
        - Os métodos de avaliação do desfecho foram os mesmos nos dois grupos.
        - Os animais foram selecionados aleatoriamente durante a avaliação dos desfechos (usar perguntas-guia do item 6).
    - “O avaliador do desfecho não foi cegado, mas os revisores julgam que não é provável que o desfecho seja influenciado pela falta de cegamento? (ex..: mortalidade)” (Sim / Não / Incerto).
    - Informações adicionais:
        - Este item precisa ser avaliado para cada desfecho principal.

8. Os desfechos com dados incompletos foram adequadamente abordados?
    - PG 8.1: Todos os animais foram incluídos nas análises? (Sim / Não/ Incerto).
    - PG 8.2: As razões para falta de dados não provavelmente não estavam relacionadas ao desfecho real (ex.: falha técnica)? (Sim / Não / Incerto).
    - PG 8.3: Os dados de desfecho faltantes estão equilibrados entre os grupos, com razões semelhantes para a falta de dados entre os grupos? (Sim / Não / Incerto).
    - PG 8.4: A imputação de dados faltantes foi feita utilizando métodos apropriados? (Sim / Não / Incerto).

9. O estudo está livre de relato de desfecho seletivo?

- PG 9.1: O protocolo do estudo estava disponível e todos os desfechos primários e secundários pré-específicados do estudo foram relatados no manuscrito atual? (Sim / Não / Incerto).
- PG 9.2: O protocolo do estudo não estava disponível, mas ficou claro que o relato publicado incluiu todos os desfechos esperados (ou seja, comparando-se as seções de métodos e resultados)? (Sim / Não / Incerto).
- Informações adicionais:
    - Relato seletivo de desfechos:
        - Nem todos os desfechos primários pré-especificados foram relatados.
        - Um ou mais desfechos primários foram relatados usando medidas, métodos de análise ou subconjuntos de dados (ex.: sub-escalas) que não foram pré-especificadas no protocolo.
        - Um ou mais desfechos primários não foram pré-especificados (a menos que clara justificativa para o relato dado – ex.: efeitos adversos inesperados).
        - O relato do estudo falhar em incluir resultados de um desfecho-chave que seria esperado relatar para tal estudo.

10. O estudo aparentemente está livre de outros problemas que poderiam resultar em alto risco de viés?
    - PG 10.1: O estudo foi livre de contaminação (combinação de drogas)? (Sim / Não / Incerto).
    - PG 10.2: O estudo foi livre de influência inapropriada de financiadores ou patrocinadores? (Sim / Não / Incerto).
    - PG 10.3: O estudo foi livre de erros nas unidades de análises (unidades experimentais)? (Sim / Não / Incerto).
    - PG 10.4: Riscos de viés específicos ao desenho experimental estavam ausentes? (Sim / Não / Incerto).
    - PG 10.5: Novos animais foram adicionados ao grupo controle e experimental para substituir perdas da população original? (Sim / Não / Incerto).
    - Informações adicionais
        - A relevância das perguntas-sinalizadoras (Tabela 3) depende do experimento. Os revisores precisam julgar por si próprios quais itens podem causar viés nos resultados e devem ser avaliados.
        - Contaminação / combinação de drogas
            - Experimentos nos quais os animais recebem, além da droga de intervenção – tratamento adicional ou drogas que podem influenciar ou causar viés no resultado.
        - Erros na unidade de análise:

- Intervenções em diferentes partes do corpo em um mesmo participante (ex.: um olho experimental, outro olho controle).
- Todos os animais recebendo a mesma intervenção estão na mesma gaiola, mas a análise é conduzida como se cada animal fosse uma única unidade experimental.

- Vieses específicos ao desenho experimental:
  - Desenho cruzado (crossover) inadequado (intervenção sem efeito temporário ou doença não estável ao longo do tempo).
  - Desenho cruzado (crossover) com risco de efeito residual (carry-over effect).
  - Desenho cruzado (crossover) com apenas dados do primeiro período disponível.
  - Desenho cruzado (crossover) em que muitos animais não recebem o segundo tratamento (ou o tratamento seguinte) devido ao alto número de perdas ou pela duração do estudo.
  - Desenho cruzado (crossover) em que todos os animais recebem a mesma ordem de intervenções.
  - Estudo de braços múltiplos em que as mesmas comparações de grupos não são relatadas para todos os desfechos (relato de desfecho seletivo).
  - Estudo de braços múltiplos em que diferentes braços são combinados (todos os dados deveriam ser apresentados por grupo).
  - Ensaio randomizado por cluster não levando em conta o agrupamento durante as análises estatísticas (erro de unidade experimental).
  - Desenho cruzado (crossover) em que a análise pareada dos resultados não é levada em consideração.

Validity assessment

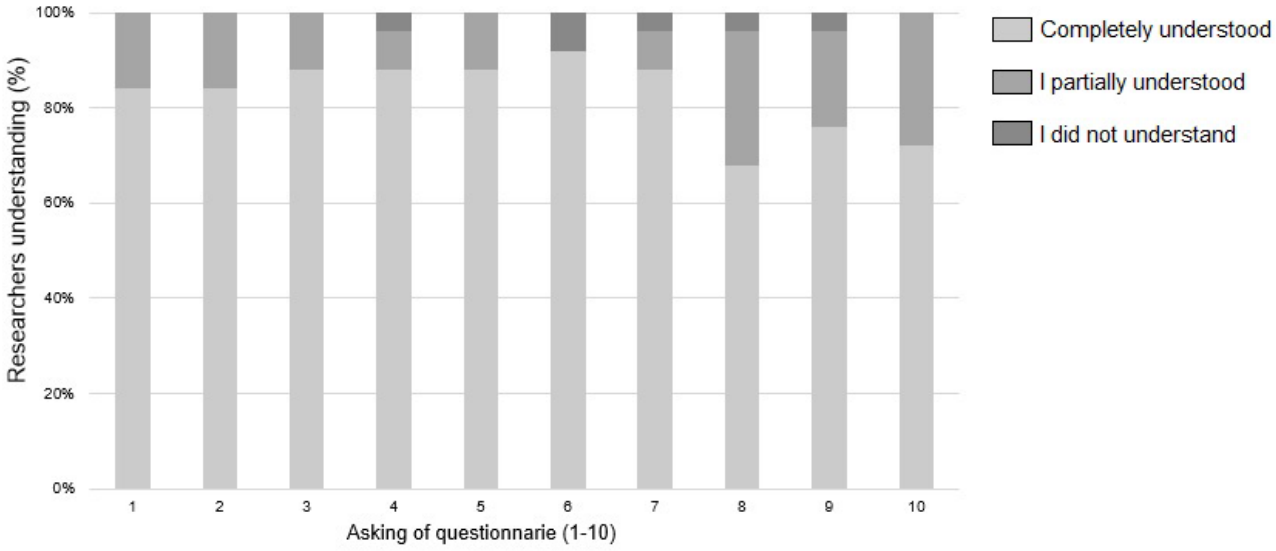

Fig. 2. The x-axis represents questions 1–10, and the y-axis the percentage of understanding for each question.

During validity assessment, most items at the translated version of the SYRCLE RoB tool were completely understood by the evaluators (Figure 2). However, some uncertainties and doubts were reported. A few examples include:

• Need to explain better the use of “uncertain” and “no – high risk of bias”: Some evaluators were unsure as to whether ‘uncertain’ could be applied to when a finding was not reported in the studies and whether findings not being reported could be considered a high risk of bias. Regarding question 2 (Selection bias – Baseline characteristics), it was suggested to use keywords that normally describe certain situations in English.

• The reviewers suggested making question 8 (Attrition Bias - Incomplete Results) completer and more adequate: determine the number of animals described in the methods and check the tables and legends if all animals were used for analyses. The terms “reinclusion for analysis” and “incomplete result” were highlighted as confusing.

Reliability evaluation

In the pre-training evaluation, the overall agreement between reviewers was calculated as 35.45% (Table 3). Four items have had an agreement rate below 10%,

which are related to a common mistake on risk of bias analysis of preclinical research: attributing high risk of bias for items that are not disclosed in an article (whereas the correct procedure would have been to score “uncertain” for these cases”). Confirming it, the use of the “uncertain” scoring option in the pre-training session was discrepant among reviewer, being 6.36% for Reviewer #1 and 53.64% Reviewer #2 (Table 4). The reduced use of “uncertain” calls attention, as this is a the most commonly used scoring option in many systematic reviews, reflecting the average poor reporting quality of preclinical studies. The Cohen’s kappa index was estimated at 0.18, indicating a slight agreement between reviewers. The discrepancies and the low agreement rates (both percentual and through the kappa index) indicates that the tool alone is not sufficient to assure a reliable risk of bias assessment.

In the post-training evaluation, the Cohen’s kappa index remained at 0.18 (slight agreement), which would indicate a lack of effect of the training on the understanding and application of the SYRCLE RoB tool. However, a deeper look into the data demonstrate some positive effects. The overall agreement rates increased from 35.45% to 53.33%. Regarding the agreement rates in individual items, items from 1 to 7 had higher scores (44.44% to 88.89%, average 76.19%). However, items 8, 9 and 10 resulted in and agreement rates of 0%. This denotes that the training improved the overall understanding and usability of the SYRCLE RoB tool but introduced a systematic error on the understanding of the three last items (which are more subjective in nature). By recalculating the Cohen’s kappa index considering items 1 to 7, a result of 0.47, improving the agreement status to moderate agreement.

Table 3. Agreement rates among reviewers

	Pre-training	Post-training
Item 1	27.27%	66.67%
Item 2	45.45%	44.44%
Item 3	0.00%	88.89%
Item 4	0.00%	88.89%
Item 5	0.00%	88.89%
Item 6	0.00%	77.78%
Item 7	9.09%	77.78%
Item 8	100.00%	0.00%
Item 9	90.91%	0.00%
Item 10	81.82%	0.00%

Overall agreement	35.45%	53.33%
Cohen's Kappa	0.18	0.18
Interpretation	Slight agreement	Slight agreement
Agreement - Items 1-7		0.74
Cohen's Kappa - 1-7		0.47
Interpretation		Moderate agreement

Agreement analyses restricted to items 1 to 7 were performed only in the post-training analysis.

Table 4. Proportion of answers per reviewer

	Pre-training		Post-training	
	Rev1	Rev2	Rev1	Rev2
Low risk of bias	38.18%	45.45%	8.89%	46.67%
Uncertain	6.36%	53.64%	86.67%	44.44%
High risk of bias	55.45%	0.91%	4.44%	8.89%

DISCUSSION

This study aimed to translate the SYRCLE RoB tool into Brazilian Portuguese. The method used in the present study enabled translation for later cross-cultural adaptation and validation of SYRCLE RoB tool for Brazilian culture. The questionnaire will allow researchers in Brazil to assess with greater ease the methodological quality of studies that will be included in systematic reviews of animal studies.

After the initial steps and translation back to the original language, the creators evaluated it, a new translation with adjustments was performed. To contact the authors, we collaborated with a member of the Brazilian Reproducibility Initiative in Preclinical Systematic Review and Meta-Analysis, a group of researchers that aims to increase the development of systematic reviews and meta-analyses in animals. In our study, the semantic, idiomatic, and grammatical equivalences of some items were necessary, consistent with the study reported by Santos et al. 2020, which, in order to guarantee the meaning of the original language, maintained semantic equivalence.

In the study carried out by Hooijmans et al. (2014) the assessment of interobserver variability in the items calculated using the kappa statistic was that items 8 (1.0) and 10 (1.0) showed perfect agreement while item 9 (0.64) showed

substantial agreement. The researchers applying the tool understood most of the tool well; however, since questions 8 and 9, did not reach the 80% level of understanding, they willrequire adjustments for Portuguese. Some suggestions, such as clarifying the “primary results” and how to address the lack of results, were made to increase clarity. Hooijmans et al. also reports that the assessment of reporting bias, item 9, in the original version, is a difficult item to assess in animal intervention studies. It is not commonly registered, but there are bases such as Preclinicaltrials.eu (https://preclinicaltrials.eu/) and the Animal Study Registry (https://www.animalstudyregistry.org) where these protocols can be registered.

In the original tool, the answer "yes" indicates a low risk of bias, “no” indicates a high risk of bias, and “uncertain” indicates an uncertain risk of bias. The researchers mostly commented that the three options confused them, but mainly reported having doubts about marking “uncertain” and “no high risk of bias” and stated that “the uncertain term should be better clarified as to your use.” This is not related to a translation problem, but rather to the way of responding to the risk of bias analysis. They left questions such as “should “uncertain” be used only when it was done? Why was it not explained?” Another question that also needed clarification was “if some items of the tool seem to not apply to some articles, more items in the article such as “does not apply” should be added”. Other translation studies went through similar processes in which adaptations of the questionnaires were necessary according to the region in which they will be applied (Claro et al. 2011; Pereira et al. 2011), which reinforces our findings.

Although all items are important, some may be more difficult to assess or have greater variability in interpretation and other items may be more nonspecific or less straightforward, which may affect the reliability of assessing risk of bias. Some items that can be considered less reliable are Detection Bias, where evaluating whether the evaluators were blind to the treatment group can be subjective, the lack of blinding can introduce bias in the results, the Attrition Bias item, where to evaluate loss of data or exclusion of animals from the study can be subjective, lack of clear information about dropout can make assessment of this item difficult, and the Reporting Bias item, where determining whether all results have been reported can be complicated, and selective publication of results may affect the interpretation of the findings. (Hooijmans et al. 2014)

These items require careful reading and interpretation, which may lead to variations in assessment. Therefore, it is important that authors of systematic reviews discuss and justify their decisions to ensure greater reliability in assessing the risk of bias.

The evaluators suggested some modifications regarding the judgment of each item of the questionnaire; therefore, the validation process of the Brazilian Portuguese version of the SYRCLE RoB tool will continue from the translated version reported in this study.

Conclusion

The SYRCLE-RoB instrument translated into Brazilian Portuguese showed moderate reliability, which can be considered valid for application in Portuguese. Only items 8 and 9 required greater attention due to the percentage of understanding not reaching 80%. However, item 8 may raise doubts when the data is not explicit in the text. On the other hand, in item 9, it is necessary to clarify the primary result.

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ANEXO 3- NORMAS DE PUBLICAÇÃO DA REVISTA BMC MEDICAL RESEARCH METHODOLOGY

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports
- list the full names and institutional addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
    - Large Language Models (LLMs), such as ChatGPT, do not currently satisfy our authorship criteria. Notably an attribution of authorship carries with it accountability for the work, which cannot be effectively applied to LLMs. Use of an LLM should be properly documented in the Methods section (and if a Methods section is not available, in a suitable alternative part) of the manuscript.
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used

- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication
- Availability of data and materials
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements
- Authors' information (optional)

Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

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Manuscripts reporting studies involving human participants, human data or human tissue must:

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- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

Studies involving animals must include a statement on ethics approval and for experimental studies involving client-owned animals, authors must also include a statement on informed consent from the client or owner.

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If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

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If your manuscript contains any individual person's data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

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If your manuscript does not contain data from any individual person, please state "Not applicable" in this section.

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All manuscripts must include an 'Availability of data and materials' statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Authors are also encouraged to preserve search strings on searchRxiv https://searchrxiv.org/, an archive to support researchers to report, store and share their searches consistently and to enable them to review and re-use existing searches. searchRxiv enables researchers to obtain a digital object identifier (DOI) for their search, allowing it to be cited.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

- The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].
- The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
- Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

- The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].
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More examples of template data availability statements, which include examples of openly available and restricted access datasets, are available here.

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Hao Z, AghaKouchak A, Nakhjiri N, Farahmand A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare. 2014. http://dx.doi.org/10.6084/m9.figshare.853801

With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].[Reference number]

If you wish to co-submit a data note describing your data to be published in *BMC Research Notes*, you can do so by visiting our submission portal. Data notes support open data and help authors to comply with funder policies on data sharing. Co-published data notes will be linked to the research article the data support (example).

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Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

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

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

Always use footnotes instead of endnotes.

References

Examples of the Vancouver reference style are shown below.

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Article within a journal

Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

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Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.

Article within a journal supplement

Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

OnlineFirst chapter in a series (without a volume designation but with a DOI)

Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

Complete book, authored

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

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