RAUL SANTOS ALVES

EFEITOS DE PEPTÍDEOS OBTIDOS DE ANIMAIS NO PROCESSO DE CICATRIZAÇÃO DE FERIDAS EM MODELOS PRÉ-CLÍNICOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

Orientadora: Reggiani Vilela Gonçalves

Coorientadores: Mariáurea Matias Sarandy Souza
Sirlene Souza Rodrigues Sartori
Leandro Licursi de Oliveira

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RESUMO

ALVES, Raul Santos, D.Sc., Universidade Federal de Viçosa, março de 2022. **Efeitos de peptídeos obtidos de animais no processo de cicatrização de feridas em modelos pré-clínicos.** Orientadora: Reggiani Vilela Gonçalves. Coorientadores: Mariáurea Matias Sarandy Souza, Sirlene Souza Rodrigues Sartori e Leandro Licursi de Oliveira.

A busca por um tratamento eficaz para feridas cutâneas tem como objetivo promover o fechamento rápido e eficaz da ferida e a formação de uma cicatriz forte e resistente. O tratamento de feridas cutâneas pode variar de acordo com a profundidade e extensão das lesões. Em geral, os cuidados incluem a limpeza da área e o uso de medicamentos ou até mesmo curativos especiais. No entanto, os tratamentos atuais têm se mostrado ineficazes no controle da infecção e da dor, o que limita o processo de cicatrização de feridas. Então, o objetivo deste trabalho foi investigar o efeito de peptídeos obtidos de animais, em especial da pele de rã-touro *Lithobates catesbeianus* (Shaw, 1802), no processo de cicatrização de feridas. Inicialmente, foi realizada uma revisão sistemática seguindo as diretrizes PRISMA, para investigar as evidências pré-clínicas de peptídeos animais na cicatrização de feridas cutâneas. A plataforma SYRCLE's RoB tool foi utilizada para análise de viés e qualidade metodológica dos estudos. Foram selecionados trinta estudos para extração dos dados. Em geral, os peptídeos animais foram eficazes em acelerar a cicatrização de feridas cutâneas por atuar na estimulação da proliferação celular, neoangiogênese, colagenogênese, reepitelização e contração da ferida. No entanto, observamos um alto risco de viés associado à caracterização limitada dos protocolos experimentais. Em seguida, foi realizada a investigação de uma fração peptídica isolada do hidrolisado de tripsina da pele de rã-touro no processo de cicatrização de feridas, utilizando análises *in vitro* e *in vivo*. Foram identificadas 71 sequências peptídicas relacionadas com proteínas desta espécie. Nos testes *in vitro* utilizando células de murinos, os peptídeos da fração investigada foram eficazes em promover a proliferação celular. No experimento *in vivo*, a fração peptídica foi eficiente em promover a contração da ferida, aumentar a proliferação celular e a síntese de colágeno, bem como reduzir a resposta inflamatória e o estresse oxidativo. Em conclusão, a fração peptídica isolada do hidrolisado de tripsina da pele da rã-touro foi eficiente no controle do processo inflamatório e consequentemente na promoção do fechamento rápido e eficaz da ferida.

Palavras-chave: Proliferação. Hidrólise enzimática. Cicatrização. Revisão sistemática.

ABSTRACT

ALVES, Raul Santos, D.Sc., Universidade Federal de Viçosa, March, 2022. **Effects of peptides obtained from animals on the wound healing process in preclinical models.** Adviser: Reggiani Vilela Gonçalves. Co-advisers: Mariáurea Matias Sarandy Souza, Sirlene Souza Rodrigues Sartori, and Leandro Licursi de Oliveira.

The search for an effective treatment for skin wounds aims to promote fast and effective wound closure and the formation of a strong and resistant scar. The treatment of skin wounds can vary according to the depth and extent of the lesions. In general, care includes cleaning the area and using medications or even special dressings. However, current treatments have been shown to be ineffective in controlling infection and pain, which limits the wound healing process. Therefore, the aim of this study was to investigate the effect of peptides obtained from animals, especially peptides from the skin of bullfrog *Lithobates catesbeianus* (Shaw, 1802), in the wound healing process. Initially, a systematic review was performed following the PRISMA guidelines to investigate the preclinical evidences of animal peptides in cutaneous wound healing. The SYRCLE's RoB tool platform was used for the analysis of bias and methodological quality of the studies. Thirty studies were selected for data extraction. In general, animal peptides were effective in accelerating the healing of skin wounds by acting in the stimulation of cell proliferation, neoangiogenesis, colagenogenesis, reepithelialization, and wound contraction. However, we observed a high risk of bias associated with limited characterization of experimental protocols. Then, the investigation of a peptide fraction isolated from trypsin hydrolysate of bullfrog skin in the wound healing process was carried out, using *in vitro* and *in vivo* analyses. 71 peptide sequences related to proteins of this species were identified. In *in vitro* tests using murine cells, the peptides of the investigated fraction were effective in promoting cell proliferation. In the *in vivo* experiment, the peptide fraction was efficient in promoting wound contraction, increasing cell proliferation and collagen synthesis, as well as reducing the inflammatory response and oxidative stress. In conclusion, the peptide fraction isolated from trypsin hydrolysate of bullfrog skin was efficient in controlling the inflammatory process and consequently promoting fast and effective wound closure.

Keywords: Proliferation. Enzymatic hydrolysis. Healing. Systematic review.

SUMÁRIO

1. INTRODUÇÃO GERAL

A pele ocupa a região mais superficial e periférica do corpo, exercendo importantes funções para o organismo como proteção, termorregulação, controle do balanço hidroeletrolítico e diversas funções metabólicas, por isso a manutenção da sua integridade é um processo de fundamental importância para a homeostase do organismo (MITTAG et al., 2017).

A pele é organizada em camadas distintas e possui origem embriológica dupla, sendo a camada de revestimento mais superficial a epiderme (de origem ectodérmica) e a derme (de origem mesodérmica) (JUNQUEIRA & CARNEIRO, 2017). Na porção mais profunda da pele, em continuidade com a derme, encontra-se a hipoderme ou tecido subcutâneo. A hipoderme é uma camada que gera muita controvérsia, pois possui origem mesodérmica e alguns autores a consideram como parte integrante da pele, sendo a derme uma camada intermediária e a hipoderme a camada mais profunda (ICHIJO et al., 2017).

A epiderme é formada por um tecido epitelial estratificado pavimentoso queratinizado, contendo uma camada de células proliferativas e várias camadas de células diferenciadas, com predominância de queratinócitos, e outras células como melanócitos, células de Langerhans e células de Merkel (SOTIROPOULOU & BLANPAIN, 2012). A derme é composta por um tecido conjuntivo frouxo e conjuntivo denso não modelado, constituída por uma variedade de células e grande quantidade de fibras de colágeno e elastina (JUNQUEIRA & CARNEIRO, 2017). Nesta camada, também estão presentes os vasos sanguíneos, um grande número de terminações nervosas e os anexos cutâneos, como os folículos pilosos, glândulas sebáceas e glândulas sudoríparas (RITTIÉ, 2016). O tecido adiposo é o principal constituinte da hipoderme, com um número variável de adipócitos e feixes irregulares de fibras de colágeno interpostos com fibras elásticas (CUNHA et al., 2017).

A perda da estrutura anatômica normal da pele e sua função, é chamada de ferida ou úlcera (FIKRU et al., 2012). As feridas podem ser classificadas de acordo com o processo de cicatrização (primeira ou segunda intenção), na cicatrização por primeira intenção, existe uma perda mínima do tecido, podendo as margens serem facilmente aproximadas, resultando em uma recuperação mais rápida (WELEDJI, 2017). Na cicatrização por segunda intenção ocorre grande perda tecidual, com um número elevado de células mortas e ruptura de vasos sanguíneos, apresentando margens afastadas, onde não há possibilidade de aproximação das bordas, ocasionando uma recuperação mais lenta (SINGH; YOUNG; MCNAUGHT, 2017). Este tipo de ferida tem sido amplamente usado nos modelos experimentais, pois permite avaliar todas as

fases do processo cicatricial e fornece quantidade de tecido suficiente para o melhor entendimento do processo de regeneração da derme (CHETTER et al., 2019).

O processo de cicatrização compreende uma série de estágios complexos marcados por eventos celulares característicos, interdependentes e simultâneos, que pode ser dividido em três fases: inflamação, proliferação e remodelação tecidual (GUSHIKEN et al., 2021).

A primeira fase do processo de cicatrização é marcada principalmente pela migração de células do sistema imune para a área da lesão, facilitada pela vasodilatação, aumento da permeabilidade vascular e liberação de substâncias quimiotáticas pelas plaquetas (SZWED & SANTOS, 2016). Os neutrófilos são as primeiras células de defesa a migrarem para o tecido e são responsáveis pelo desbridamento do tecido lesionado, fagocitose de agentes infecciosos e liberação de várias substâncias antimicrobianas (BALSA & CULP, 2015). Os monócitos são atraídos para o local da lesão pelos produtos da degradação da matriz extracelular e fragmentos de colágeno, fibronectina e trombina, onde diferenciam-se em macrófagos no tecido (YUAN et al., 2015). Os macrófagos são células regulatórias importantes no processo de cicatrização, desempenhando papel fundamental no final do desbridamento iniciado pelos neutrófilos, na fagocitose de patógenos e liberação de vários fatores de crescimento (SZWED & SANTOS, 2016). Além disso, estas células produzem e secretam óxido nítrico e espécies reativas de oxigênio, que são essenciais para a proteção contra bactérias e outros microrganismos invasores (KIM et al., 2019). No entanto, níveis muito elevados destas moléculas podem provocar danos as células presentes no tecido cicatricial e comprometer o processo de reparo (SIES; BERNDT; JONES, 2017). Os macrófagos podem exibir diferentes fenótipos durante o processo de cicatrização, sendo os macrófagos M1 caracterizados pela produção de citocinas pró-inflamatórias nas fases iniciais do processo de reparo, enquanto o tipo M2 é caracterizado pela produção de citocinas anti-inflamatórias, atuando na proliferação celular, angiogênese e deposição de matriz nos estágios finais do processo (KOH & DIPIETRO, 2011). A transição da fase inflamatória para a fase proliferativa, depende da efetividade dos macrófagos na estabilização do microambiente, segregação de vários agentes quimiotáticos, citocinas e fatores de crescimento que ativam elementos celulares da fase subsequente (KIM et al., 2019).

Na fase proliferativa, os fibroblastos ativos sintetizam colágeno, glicosaminoglicanos e fibronectina, promovendo modificações na composição da matriz derivada de plaquetas e do plasma, dando origem ao tecido de granulação (BALSA & CULP, 2015). Esse tecido muito frágil é composto por diferentes tipos de células, pequenos vasos sanguíneos e uma matriz extracelular composta principalmente por fibras colágenas do tipo III, que ajuda a proteger a ferida de mais danos (LANDÉN; LI; STÅHLE, 2016). Outro processo importante que acontece

nesta fase, é a migração de queratinócitos para o local da lesão, com o intuito de reestabelecer a epiderme, minimizando a perda de fluidos e invasão bacteriana (ISAAC et al., 2010).

A fase de remodelação é caracterizada por intensa modificação nos componentes da matriz extracelular. Nesta fase o tecido de granulação altamente celular e rico em fibras colágenas tipo III sofre uma diminuição no número de células endoteliais, miofibroblastos e células inflamatórias por apoptose, e o colágeno tipo III é substituído pelo tipo I, conferindo a matriz força e resistência (LANDÉN; LI; STÅHLE, 2016).

Quando o processo de cicatrização é interrompido ou retardado, a ferida pode tornar-se crônica, causando ao indivíduo desconforto e dor, e podendo evoluir para casos mais graves, como amputação, sepse e até mesmo a morte (STANOJCIC; VINAIK; JESCHKE, 2018). Desta forma, as feridas crônicas representam um sério problema de saúde pública, pois muitas vezes apresentam um tratamento longo e dispendioso (GUEST et al., 2015).

Devido aos elevados gastos anuais na área da saúde para o tratamento e prevenção das feridas cutâneas e levando em consideração que os métodos atuais não têm sido efetivos em vários casos, têm crescido as buscas por tratamentos alternativos para melhorar o processo cicatricial (GUEST et al., 2015). Neste contexto, podemos destacar os ranídeos que vêm despertando o interesse de vários pesquisadores para o desenvolvimento de compostos bioativos com potencial terapêutico (RINALDI, 2002). No gênero *Lithobates*, existem diversos peptídeos antimicrobianos isolados a partir da pele destes animais, pertencentes a diferentes famílias de peptídeos como brevenina-1, esculentina-1, esculentina-2, palustrina-2, ranaciclina, ranalexina, ranatuerina-1, ranatuerina-2 e temporina (CONLON, 2008). Novos peptídeos ainda estão sendo descobertos, como o catesbeianin-1, isolado a partir da pele de rã-touro, que apresenta atividade antimicrobiana e baixa toxicidade para linhagens celulares de camundongos e humanos, sendo um potencial agente terapêutico para o tratamento de feridas infectadas (XU et al., 2017).

Diante do exposto, a presente tese teve como objetivo avaliar o efeito de peptídeos obtidos da pele de rã-touro no processo de reparo cutâneo. A tese foi dividida em dois capítulos em formato de artigo científico. No primeiro capítulo, é apresentada uma revisão sistemática sobre a utilização de peptídeos de origem animal no processo reparo cutâneo. Essa revisão sistemática foi utilizada para sintetizar a evidência científica sobre os peptídeos de origem animal que estão sendo pesquisados como potenciais agentes terapêuticos em estudos pré-clínicos, a fonte biológica destes peptídeos e a qualidade metodológica dos estudos experimentais. O segundo foi um estudo experimental *in vitro* e *in vivo*, no qual foi avaliado o efeito de uma fração peptídica isolada do hidrolisado de tripsina da pele de rã-touro no processo

de reparo de feridas cutâneas de segunda intenção em camundongos. Na parte *in vitro*, foi observado a ação destes compostos na proliferação celular de macrófagos e fibroblastos. Na parte *in vivo*, foram feitas as seguintes análises: contração da ferida, histologia do tecido cicatricial, imunofluorescência, expressão gênica, marcadores do estresse oxidativo e atividade de enzimas antioxidantes.

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2. ARTIGO I

PEPTIDES FROM ANIMAL ORIGIN: A SYSTEMATIC REVIEW ON BIOLOGICAL SOURCES AND EFFECTS ON SKIN WOUNDS

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Abstract

Background

Skin wounds are closely correlated with opportunistic infections and sepsis risk. Due to the need of more efficient healing drugs, animal peptides are emerging as new molecular platforms to accelerate skin wound closure and to prevent and control bacterial infection.

Aim

The aim of this study was to evaluate the preclinical evidence on the impact of animal peptides on skin wound healing. In addition, we carried out a critical analysis of the studies' methodological quality.

Main methods

This systematic review was performed according to the PRISMA guidelines, using a structured search on the PubMed-Medline, Scopus and Web of Science platforms to retrieve studies published until August 25, 2020 at 3:00 pm. The studies included were limited to those that used animal models, investigated the effect of animal peptides with no association with other compounds on wound healing and that were published in English. Bias analysis and methodological quality assessments were examined through the SYRCLE's RoB tool.

Results

Thirty studies were identified using PRISMA workflow. In general, animal peptides were effective in accelerating skin wound healing, especially by increasing cellular proliferation, neoangiogenesis, colagenogenesis and reepithelialization. Considering standardized methodological quality indicators, we identified a marked heterogeneity in research protocols and a high risk of bias associated with limited characterization of the experimental designs.

Conclusion

Animal peptides show a remarkable healing potential with biotechnological relevance for regenerative medicine. However, rigorous experimental approaches are still required to clearly delimit the mechanisms underlying the healing effects and the risk-benefit ratio attributed to peptide-based treatments.

Keywords: Natural products, Bioactive peptides, Wound healing.

Introduction

Due to the disruption of innate defense mechanisms, skin wounds are a serious risk factor for opportunistic infections, bacteremia and sepsis [1–3]. In the United States, recent estimates indicate that at least US$25 billion are spent annually in the treatment of 6.5 million patients with chronic wounds [4]. The treatment of skin wounds is a challenge task, especially considering that the available treatments have limited spectrum of action on cellular and molecular mechanisms involved in tissue repair [5–9]. Skin wound healing requires a series of cellular and molecular interdependent events in order to restore tissue integrity after trauma [5]. This process is mediated by growth factors, cytokines and resident and transitory cells, and is organized in phases involving inflammation, cell proliferation and tissue remodeling/maturation [6]. In the inflammatory phase, immune cells such as neutrophils and macrophages migrate to the lesion area to remove tissue debris, promote antimicrobial defenses and trigger cell proliferation [7]. The proliferative phase is marked by intense cellular activity and different cells migration to the wound bed. At this stage, fibroblasts form the granulation tissue, composed of cells and a network of blood vessels, re-establishing regional circulation [8]. The remodeling phase corresponds mainly to changes in the extracellular matrix of the scar tissue, where most type III collagen fibers are progressively replaced by type I fibers, which are more resistant and abundant in intact skins [9]. Two subsets of macrophages (M1 or M2) are commonly identified in this process, exerting complementary effects in early and late stages of tissue repair [10]. M1 macrophages are activated by interferon gamma (IFN-γ), exerting potent nitric oxide-mediated antimicrobial effects and pro-inflammatory responses in the initial stages of tissue repair [11]. As an overlapping event between proliferative and remodeling phases, M2 macrophages are activated by cytokines such as IL-4, IL-10 or IL-13 [11]. These cells play an essential role on the effective resolution of the inflammation, mainly through angiogenesis and extracellular matrix resorption and remodeling [10,11].

Wound healing is a complex and time sensitive process often impaired by several factors such as infections, metabolic comorbidities (i.e., diabetes, dyslipidemia, malnutrition and circulation disorders such as thrombosis, atherosclerosis, and hemorrhage), as well as the presence of foreign bodies that may delay wound healing by stimulating a chronic inflammatory response [1]. In a continuous effort to improve the pharmacological management of skin wounds, the screening of natural molecules capable of modulating the biological processes involved in tissue repair are proposed as a rational and promising strategy for the biotechnological development of more efficient healing drugs [12]. In order to achieve greater therapeutic efficacy, the search for new molecules also aim at overcoming current limitations of healing drugs, especially the technical difficulty in obtaining the active metabolites, the high cost of drug production, the formation of hypertrophic scars, and the risk of selecting treatment-resistant microorganisms, an aspect that represents a global concern [13,14].

Due to its antimicrobial, immunomodulatory, pro-mitotic, colagenogenic and neoangiogenic potential, animal peptides are suggested as promising agents for new therapeutic approaches in skin wound treatment [1,12,15,16]. Besides, their molecular abundance, low cost of isolation techniques, high molecular stability, and their broad spectrum of biological properties are also encouraging characteristics. However, the main animal peptides, physicochemical characteristics of the bioactive molecules, effective doses and routes of administration are not completely understood. Considering that current evidence is based on fragmented data, it is unclear whether and to what extent animal peptides are effective in the skin wounds treatment. In addition, it is currently difficult to understand the metabolic pathways and mechanisms of actions activated by these peptides during the skin repair. Thus, we used the systematic review framework to evaluate preclinical evidence on the impact of animal peptides on skin wound healing. In addition to characterize the biological sources of these

peptides and its chemical sequences, the methodological quality of all studies reviewed was critically evaluated.

Methodology

Retrieval of research records

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) workflow [17], which is used as a guide for study selection, screening and eligibility. Studies were selected through an advanced search on the platforms PubMed-Medline (https://www.ncbi.nlm.nih.gov/pubmed), Scopus (https://www.scopus.com/home.uri) and Web of Science (https://www.webofknowledge.com), on August 25, 2020 at 3:00 pm. We used a comprehensive search strategy for retrieving all relevant studies, with a primary search in electronic databases and a secondary search in the reference lists from all relevant studies identified in the primary search. For all databases, the search filters were based on three complementary levels: (i) intervention: animal peptides; (ii) biological process: wound healing; and (iii) target organ: skin. The PubMed-Medline platform filters were built using hierarchical distribution of MeSH (Medical Subject Headings) terms to retrieve the indexed studies. Non-MeSH descriptors were characterized by TIAB algorithm (Title and Abstract). To identify preclinical studies, a standardized experimental animal filter was applied [18]. The search filters used for the PubMed-Medline search platform were adapted to Scopus and Web of Science databases, except for the experimental animal filter used in Scopus, which was provided by the site. The complete search strategy is shown in the supplementary file (S1 Table).

Selection of relevant studies

Only studies that met all the inclusion criteria as described below were selected: (i) *in vivo* studies using animal models; (ii) studies that investigated the effect of animal peptides with no association with other compounds on wound healing; and (iii) original studies published in English. The following studies were excluded: (i) non-animal peptides; (ii) unreported origin of peptides; (iii) investigations of other organs, pathologies or therapies; (iv) sutured wounds; (v) *in vitro* and *ex vivo* studies; (vi) unreachable studies; (vii) secondary research (i.e., literature reviews, comments, letters and editorials); and (viii) gray literature (i.e., video-audio media). When it was difficult to obtain the full text papers, the authors were requested to provide it by email.

Data extraction and management

Two independent reviewers (RSA and LSA) conducted the literature search, removed duplicated articles, and screened titles and abstracts with respect to eligibility criteria. After initial screening, full-text articles of potentially relevant studies were independently assessed for eligibility by two reviewers (RSA and LSA). The kappa test was done for the selection and data extraction (kappa=0.922). Selections were then compared, and inconsistencies were resolved in consultation with three other reviewers (MMS, RDN and RVG). Data from each study was extracted using well-defined data as follows: (i) publications characteristics (author, year of publication and country of origin); (ii) animal models (animal, strain, sex, age, weight and associated pathology); (iii) cutaneous wounds (type of lesion, site, initial area, number, and presence of infection); (iv) peptide characteristics (name, origin and amino acid sequence); (v) intervention characteristics (route of administration, concentration, vehicle, frequency and

duration); (vi) primary outcome (wound closure); and (vii) secondary outcomes (cell proliferation and differentiation, synthesis of extracellular matrix components, recruitment of inflammatory cells, neoangiogenesis, inflammatory mediators, and oxidative markers). Quantitative data related to the wound area were directly collected from the tables or the main text provided in each study. When these data were graphically represented, the values of wound area were obtained using the Image-Pro Plus 4.5 image analysis software (Media Cybernetics, MD, USA). The wound area was compared amongst experimental groups and the results were expressed in percentage of wound closure.

Bias analysis

The risk of bias was analyzed using the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) Risk of Bias (RoB) tool [19]. This instrument is based on the Cochrane Collaboration RoB Tool, which is adjusted for aspects of bias that play a specific role in animal intervention studies. The goal was to avoid discrepancies in the assessment of methodological quality in the field of animal experimentation. To increase transparency and applicability, signaling questions were answered to facilitate judgment based on the following domains: (i) sequence generation; (ii) baseline characteristics; (iii) allocation concealment; (iv) random housing; (v) blinding; (vi) random outcome assessment; (vii) incomplete outcome data; (viii) selective outcome; and (ix) other sources of bias. Two reviewers (RSA and RVG) independently assessed the risk of bias for each study; any disagreements were resolved by discussion and consensus with two other reviewers among the authors (MMS and RDN). The SYRCLE chart was built using the Review Manager 5.3 program (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration).

Results

Included studies

We found 1734 articles, of which 376 were duplicated and 1220 studies were excluded due to inadequate research theme. Among the excluded studies, 502 did not use peptides, 458 were related to other tissues, pathologies or therapies, 108 were reviews, 68 did not evaluate the wound healing process, 57 used peptides of non-animal origin, 10 were unreachable, 9 were not written in English, 3 were studies *in vitro*, 2 were comments, 1 was an *ex vivo* study, 1 was a letter and 1 was a video-audio media. The remaining 138 articles were carefully analyzed, of which 108 were excluded for not meeting the eligibility criteria (S2 Table). Thus, 30 relevant articles were selected. After reading the reference list of all selected articles, no relevant article was found. PRISMA diagram indicates the study selection process (Figure 1). The selected studies were conducted in 7 different countries, mainly China (n = 18, 60%), followed by Taiwan and United States of America (n = 4, 13% each), Portugal, Korea, India and Saudi Arabia (n = 1, 3% each).

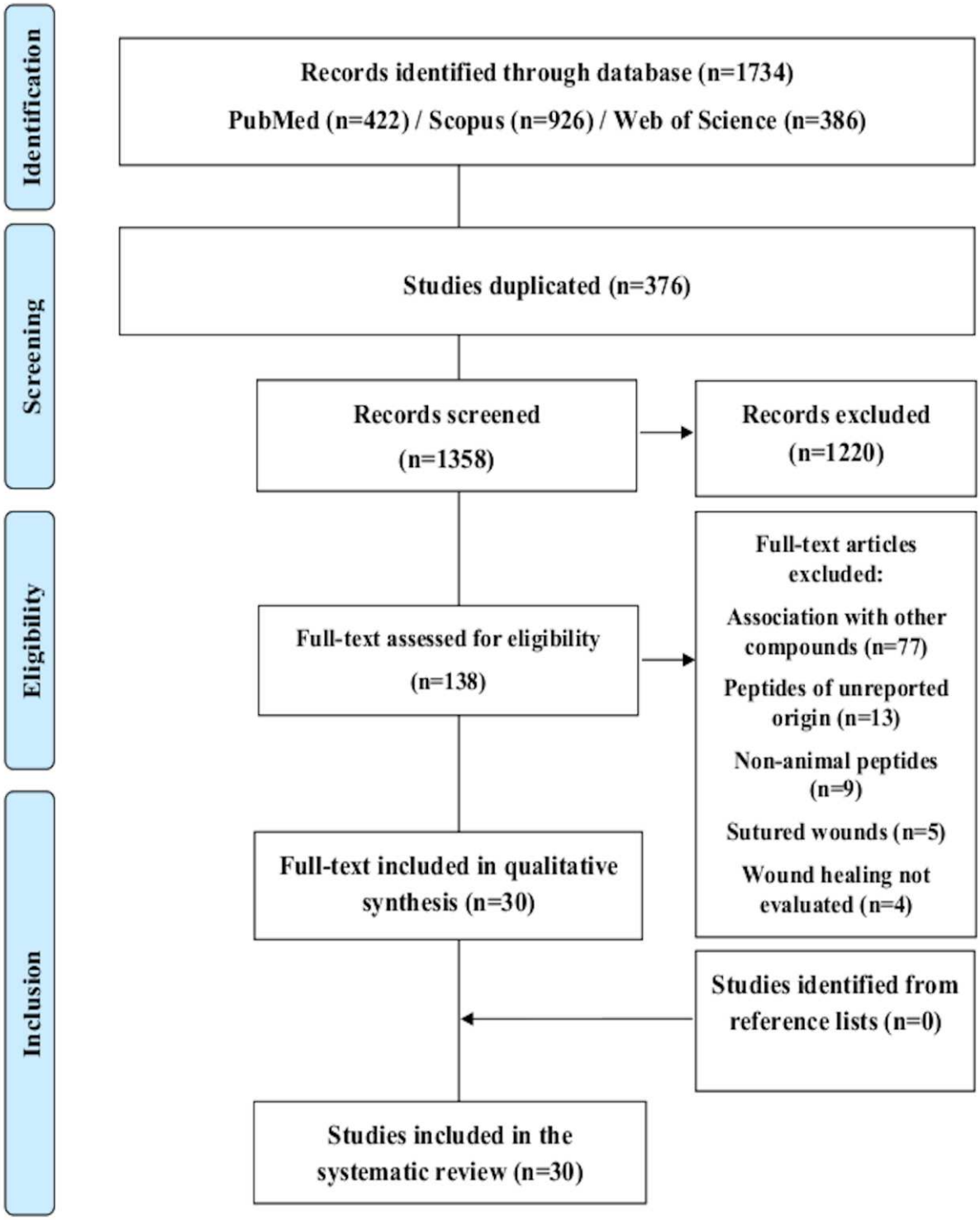

Figure 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram. The flowchart indicates the research records obtained at all standardized stages of the search process required for the development of systematic reviews and meta-analyzes. Based on the PRISMA statement (http://www.prisma-statement.org)**.**

Characteristics of preclinical models

The most used animal model was mice (n = 20, 67%), followed by rat (n = 8, 27%), pig and rabbit (n = 1, 3% each). The most used strain was Balb/C for mice (n = 6, 30%) and Sprague-Dawley for rat (n = 6, 75%), but 30% of the studies did not report this information (n = 9). Most studies included only males (n = 19, 63%), 17% used only females (n = 5), 3% used both (n = 1) and 17% did not report this data (n = 5). The age of the animals ranged from 6 to 12 weeks for mice, 6 to 43 weeks for rats and 6 weeks for pigs. This information was not reported in 57% of the studies (n = 17). Animal weight ranged from 20 to 26 g for mice, 150 to 600 g for rats and 10 to 13 kg for pigs. This information was underreported in most studies (n = 14, 47%). Most studies were performed on health animals (n = 26, 87%), 10% used diabetic models (n = 3) and 3% used ischemic model (n = 1). The main characteristics related to animal models are described in detail in S3 Table.

Characteristics of skin wounds

Most studies investigated excisional wounds (n = 27, 90%), followed by burns (n = 2, 7%) and incisional wounds (n = 1, 3%). The most used site for wounds was the back of the animal (n = 29, 97%) and 3% performed the injury on the abdomen (n = 1). The initial wound area was reported in all studies (n = 30, 100%). The number of wounds ranged from 1 to 6 per animal (n = 27, 90%) and 10% did not report this information (n = 3). *Staphylococcus aureus* and *Escherichia coli* were the microorganisms used in experiments with infected wounds (n = 5, 17%), and bacterial load concentration ranged from 2 x 10^5 to 10^{10} Colony Forming Unit (CFU). The main characteristics related to skin wounds are detailed in S4 Table.

Characteristics of animal peptides and treatments

The name and origin of the peptides used was reported in all studies (n = 30, 100%). Most of the peptides originated from amphibians (n = 11, 37%), followed by mammals and fishes (n = 8, 27% each), jellyfish, mollusk and insect (n = 1, 3% each). The amino acid sequences in these peptides were described in 63% of the studies (n = 19). The most commonly used route of peptide administration was topical (n = 20, 67%), followed by oral (n = 6, 20%), subcutaneous (n = 2, 7%), Intravenously (n = 1, 3%) and 3% evaluated two routs (topic and intraperitoneal) (n = 1). The most used vehicle was saline solution (n = 13, 43%), followed by phosphate-buffered saline (n = 10, 33%), Dulbecco's phosphate-buffered (n = 2, 7%), water (n = 2, 7%) and 10% did not report this information (n = 3). Most studies applied the intervention twice a day (n = 10, 33%), followed by once a day (n = 8, 27%), single application (n = 2, 7%), three times per day, continuous intervention, every three days and twice or every two days (n = 1, 3% each). In 20% of the studies this information was underreported (n = 6). Duration of intervention ranged from 5 to 11 days in 27% of studies (n = 8), 12 to 16 days in 10% of studies (n = 3), 22 to 26 days in 3% of the studies (n = 1), 27 to 31 days in 3% of the studies (n = 1) and 57% did not report this data (n = 17). The peptide-related characteristics and treatment protocols are described in S5-S6 Tables, respectively. Main outcome (reduction in wound size) in the treatment of skin wounds using peptides of animal origin is described in Table 1.

Table 1. Main outcome in the treatment of skin wounds using peptides of animal origin.

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[20]	Thymosin β4	Topic	Twice	5 µg/50µl	62%	7	?	?	?	?	?	?	?	?
		I.p.	Every two days	60 µg/300µl	61%									
[21]	TP508	Topic	Single	0.03 µg	47%	7	?	?	?	?	?	?	?	?
				0.1 µg	39%	10								
				0.3 µg	79%	7								
				0.4 µg	37%	10								
				1 µg	43%	10								
				1 µg	78%	7								
				3 µg	22%	7								
				5 µg	?	?								
[22]	TP508	Topic	Single	0.1 µg	?	?	?	?	?	?	53%	14	?	?
[23]	HB-107	Topic	Three times per day	100 µg/ml	63%	11	?	?	?	?	?	?	?	?
[15]	Marine collagen peptides (MCP)	Oral	Once daily	2 g/kg	76%	16	?	?	?	?	?	?	?	?

*: Results shown as a percentage of reduction in the average wound area of the groups treated with peptide compared to the control group on a given post-injury day, RF: Reference, P: Peptides. R: Route, A: Application, C: Concentration, RWS: Reduction in wound size, DA: Day analyzed, PI: Post-injury, ?: Not reported or unclear, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously, CP1: Collagen peptides bands at 10-15kDa, CP2: Collagen peptides <25kDa, Ss-SCP: *Salmo salar* skin collagen peptides, Tn-SCP: *Tilapia nilotica* skin collagen peptides.

Table 1. (Continued).

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[24]	LL37	Topic	Twice daily	10 µg	?	?	?	?	?	?	?	?	?	?
[25]	AH90	Topic	Twice daily	250 µg/ml	64%	10	?	?	?	?	?	?	?	?
[26]	Pardaxin (GE33)	Topic	?	8 mg/ml	58%	21	85%	17	?	?	?	?	?	?
[27]	Tylotoin	Topic	Twice daily	20 µg/ml	89%	10	?	?	?	?	?	?	?	?
[28]	CW49	Topic	Twice daily	200 µg/ml	64%	8	?	?	23%	8	?	?	?	?

*: Results shown as a percentage of reduction in the average wound area of the groups treated with peptide compared to the control group on a given post-injury day, RF: Reference, P: Peptides. R: Route, A: Application, C: Concentration, RWS: Reduction in wound size, DA: Day analyzed, PI: Post-injury, ?: Not reported or unclear, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously, CP1: Collagen peptides bands at 10-15kDa, CP2: Collagen peptides <25kDa, Ss-SCP: *Salmo salar* skin collagen peptides, Tn-SCP: *Tilapia nilotica* skin collagen peptides.

Table 1. (Continued).

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[29]	E1	Topic	Once daily	60 µM	92%	12	?	?	?	?	?	?	?	?
[30]	Tilapia piscidin 4 (TP4)	Topic	?	2 mg/ml	27%	19	29%	19	?	?	?	?	?	?
[12]	Tilapia piscidin 3 (TP3)	Topic	?	2 mg/ml	23%	19	44%	19	?	?	?	?	?	?
[31]	Proinsulin C	S.c.	Continuous	35 pmol/kg per minute	?	?	?	?	67%	10	?	?	?	?
[32]	Camel milk peptide (CMP)	Oral	Once daily	25 mg/kg	?	?	?	?	37%	7	?	?	?	?

*: Results shown as a percentage of reduction in the average wound area of the groups treated with peptide compared to the control group on a given post-injury day, RF: Reference, P: Peptides. R: Route, A: Application, C: Concentration, RWS: Reduction in wound size, DA: Day analyzed, PI: Post-injury, ?: Not reported or unclear, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously, CP1: Collagen peptides bands at 10-15kDa, CP2: Collagen peptides <25kDa, Ss-SCP: *Salmo salar* skin collagen peptides, Tn-SCP: *Tilapia nilotica* skin collagen peptides.

Table 1. (Continued).

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[33]	Ghrelin	S.c.	Once daily	50 nmol/kg 100 nmol/kg 200 nmol/kg	17% 0% 0%	14	?	?	?	?	?	?	0% 50% 67%	14
[34]	Epinecidin-1 (Epi-1)	Topic	Every three days	90 µg/ml 900 µg/ml 9 mg/ml	?	?	65% 65% 71%	25	?	?	?	?	?	?
[35]	Marine collagen peptides (MCP)	Topic	Once daily	?	86%	21	?	?	?	?	?	?	?	?
[1]	OM-LV20	Topic	Twice daily	0.5 nM 1 nM 2.5 nM 5 nM 10 nM 20 nM	? ? ? ? ? 50%	10	?	?	?	?	?	?	?	?
[13]	Cathelicidin-OA1	Topic	Twice daily	10 µM 20 µM 40 µM	6% 53% 66%	10	?	?	?	?	?	?	?	?

*: Results shown as a percentage of reduction in the average wound area of the groups treated with peptide compared to the control group on a given post-injury day, RF: Reference, P: Peptides. R: Route, A: Application, C: Concentration, RWS: Reduction in wound size, DA: Day analyzed, PI: Post-injury, ?: Not reported or unclear, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously, CP1: Collagen peptides bands at 10-15kDa, CP2: Collagen peptides <25kDa, Ss-SCP: *Salmo salar* skin collagen peptides, Tn-SCP: *Tilapia nilotica* skin collagen peptides.

Table 1. (Continued).

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[16]	OA-GL21	Topic	Twice daily	1 µg/ml 10 µg/ml 100 µg/ml	-3% 44% 53%	9	?	?	?	?	?	?	?	?
[36]	Cathelicidin-NV	Topic	Twice daily	200 µg/ml	91%	10	?	?	?	?	?	?	?	?
[37]	Pollock Collagen Peptide (PCP)	Oral	?	0.5 g/kg 2 g/kg	27% 48%	12	?	?	?	?	?	?	?	?
[38]	OA-FF10	Topic	Twice daily	1 µM 10 µM 100 µM	52% 52% 68%	8	?	?	?	?	?	?	?	?
[39]	Collagen peptides (CP1/CP2)	Oral	Once daily	0.3 g/kg 0.6 g/kg 0.9 g/kg	-27%/-92% -20%/-71% 47%/-17%	7	?	?	?	?	?	?	?	?

*: Results shown as a percentage of reduction in the average wound area of the groups treated with peptide compared to the control group on a given post-injury day, RF: Reference, P: Peptides. R: Route, A: Application, C: Concentration, RWS: Reduction in wound size, DA: Day analyzed, PI: Post-injury, ?: Not reported or unclear, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously, CP1: Collagen peptides bands at 10-15kDa, CP2: Collagen peptides <25kDa, Ss-SCP: *Salmo salar* skin collagen peptides, Tn-SCP: *Tilapia nilotica* skin collagen peptides.

Table 1. (Continued).

RF	P	Intervention			Main outcome*									
					Normal wound		Infected wound		Diabetic wound		Ischemic wound		Radiation + wound	
		R	A	C	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)	RWS (%)	DA (PI)
[40]	OA-GL12	Topic	Twice daily	0.1 nM 1 nM 10 nM	13% 44% 63%	10	?	?	?	?	?	?	?	?
[41]	Ot-WHP	Topic	Once daily	200 µg/ml	63%	8	?	?	?	?	?	?	?	?
[42]	Active peptides (APs)	Oral	?	0.5 g/kg 2 g/kg	60% 80%	14	?	?	?	?	?	?	?	?
[43]	Skin collagen peptide (Ss-SCP/Tn-SCP)	Oral	?	2 g/kg	59%/45%	12	?	?	?	?	?	?	?	?
[44]	Cathelicidin-DM	I.v.	Once daily	10 mg/kg	?	?	?	?	?	?	?	?	?	?

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Main biological outcomes

In general, studies identified in this review support the evidence that animal peptides exert healing properties on skin wound. Although the reports are heterogeneous, all studies (n = 30, 100%) show that animal peptides are effective in accelerating wound closure. Most studies that performed histological analysis (n = 23, 77%) reported improvement in the processes of reepithelialization and dermal regeneration, inflammatory cells recruitment, and blood vessels and collagen fibers formation. Immunohistochemical analyses were performed in 50% of the studies (n = 15), which showed the effects of peptides in the quantitative increase of myofibroblasts, inflammatory cells, blood vessel density, growth factors such as factors β-fibroblast growth factor (β-FGF), vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1), as well as the reduction of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Enzyme-linked immunosorbent assay (ELISA) was performed in 33% of the studies (n = 10), which reported a reduction in proinflammatory cytokines such as IL-6 and TNF-α, as well as an increase in growth factor VEGF and TGF-β1. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in 10% of the studies (n = 3), which highlighted the influence of peptides on the upregulation of growth factor-related genes such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and VEGF, and also on the gene related to macrophage migration inhibition factor (MIF), and down-regulation of genes related to pro-inflammatory cytokines such as IL-6 and TNF-α, as well as the expression of the CXCL5 gene. The Western blot technique was performed in 7% of the studies (n = 2), which highlighted the increased expression of angiogenic proteins such as hypoxia-inducible factor-1α**,** endothelial nitric oxide synthase and inducible nitric oxide synthase, as well as VEGF and TGF-β1. Oxidative stress analysis was performed in 7% of the studies (n = 2), in which peptides increased glutathione (GSH) level and the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT); as well as reduced the level of malondialdehyde (MDA), a lipid peroxidation marker. All relevant results involving the use of animal peptides in the treatment of skin wounds are described in Table 2.

Table 2. All relevant results reported in all studies included in the systematic review on peptides of animal origin applied in the treatment of skin wounds.

Peptide source	Outcomes	
	Increased	**Reduced**
Human [21,22,24,31]	Wound closure [21,22,24,31] Reepithelialization [24] Inflammatory cells [21,22] Blood vessels [21,22,24,31] Tensile strength [21]	Wound area [21,22,24,31] Inflammatory cells [31] IL-1β, IL-6 and TNF-α [31]
Other mammals [20,29,32,33]	Wound closure [20,29,32,33] Reepithelialization [20,29] Dermal regeneration [20,32] Inflammatory cells [32] Blood vessels [20,32,33] Collagen [20,29,32,33] SOD, CAT, GSH and MIF [32] Hexosamine [29,33] Ascorbate and Proteins [29] Tensile strength [29] Collagen contraction temperature [29] DNA, NO, VEGF and TGF-β1 [33]	Wound area [20,29,32,33] MDA, TNF-α and NF-kB [32] Lipid peroxidation [29]
Amphibian [1,13,44,16,25,27,28,36,38,40,41]	Wound closure [1,13,44,16,25,27,28,36,38,40,41] Reepithelialization [13,16,25,27,28,36,40,41] Dermal regeneration [13,16,25,27,28,36,40,41] Inflammatory cells [13,27,41] Blood vessels [28] Collagen [36,41] Myofibroblasts [25,27,36,41] MCP-1 and VEGF [36] TNF-α [36,41] TGF-β [41] TGF-β1 [13,27,36] CXCL1 and CCL2 [41] HIF-1α, eNOS and iNOS in diabetic wounds [28]	Wound area [1,13,44,16,25,27,28,36,38,40,41] Inflammatory cells [28] IL-6 and TNF-α in diabetic wounds [28]

IL: Interleukin, TNF: Tumor necrosis factor, SOD: superoxide dismutase, CAT: Catalase, GSH: Glutathione, MIF: Macrophage migration inhibitory factor, DNA: Deoxyribonucleic acid, NO: Nitric oxide, VEGF: Vascular endothelial growth factor, TGF: Transforming growth factor, MDA: Malondialdehyde, NF-kB: Transcription factor kappa-B, MCP: Monocyte chemoattractant protein, HIF: Hypoxia-inducible factor, eNOS: Endothelial nitric oxide synthase, iNOS: Inducible nitric oxide synthase, EGF: Epidermal growth factor, CRP: C-reactive protein, FGF: Fibroblast growth factor, TβR: Transforming growth factor-β receptor.

Table 2. (Continued).

Peptide source	Outcomes	
	Increased	**Reduced**
Fish [12,15,26,30,34,35,37,43]	Wound closure [12,15,26,30,34,35,37,43] Reepithelialization [12,26,34,35,37,43] Dermal regeneration [12,26,34,35,37,43] Inflammatory cells [26,34] Collagen [34,37,43] VEGF [26,30,43] EGF and TGF-β [30,37] FGF [43] bFGF [37] TβRII [37] IL-1 [30] IL-10 [43] NOD2 and BD14 [43] Hydroxyproline [37]	Wound area [12,15,26,30,34,35,37,43] Inflammatory cells [12] IL-6 [12,26,30,34] TNF [30] MCP-1 [26] TNF-α [12,26] CRP [34] CXCL5 [12] Bacterial loads [12,26,34]
Jellyfish [39]	Wound closure, Reepithelialization, Dermal regeneration, Collagen, β-FGF and TGF-β1	Wound area
Mollusk [42]	Wound closure, Reepithelialization, Dermal regeneration, CD31, EGF, FGF, TGF-β, TβRII, IL-1 and IL-10	Wound area, Inflammatory cells and Smad7
Insect [23]	Wound closure, Reepithelialization and Inflammatory cells	Wound area

IL: Interleukin, TNF: Tumor necrosis factor, SOD: superoxide dismutase, CAT: Catalase, GSH: Glutathione, MIF: Macrophage migration inhibitory factor, DNA: Deoxyribonucleic acid, NO: Nitric oxide, VEGF: Vascular endothelial growth factor, TGF: Transforming growth factor, MDA: Malondialdehyde, NF-kB: Transcription factor kappa-B, MCP: Monocyte chemoattractant protein, HIF: Hypoxia-inducible factor, eNOS: Endothelial nitric oxide synthase, iNOS: Inducible nitric oxide synthase, EGF: Epidermal growth factor, CRP: C-reactive protein, FGF: Fibroblast growth factor, TβR: Transforming growth factor-β receptor.

Reporting bias

Regarding the analysis of bias obtained with SYRCLE's RoB tool, highest risks of bias found in the studies were related to the methods used in the generation and application of the animal allocation sequence, housing procedures, and animals selection for outcome assessment. Regarding baseline similarities, 10% of the studies reported sufficient information to conclude that the distribution was balanced among the intervention and control groups at the beginning of the experiment (n = 3), and 90% did not report sufficient information on the homogeneity of the experimental models (n = 27). Regarding the measures used to blind caregivers and/or investigators, only 3% reported this information (n = 1). Considering the evaluators, two studies (7%) reported that the outcomes were collected in a blind manner, 10% reported that the evaluation was performed by independent researchers, but does not provide information on blinding (n = 3), and 83% did not report this information at all (n = 25). Regarding incomplete results adequately addressed, 77% did not report or showed unclear information (n = 23). Considering the item that evaluates whether the study is free of selective outcome reports, 50% did not make clear the expected results (n = 15). Other potential risk of bias that could compromise the evidence (i.e., additional treatment or drugs and interventions applied to different parts of the body within one participant) were found in 50% of the studies (n = 15). Results from bias analysis are shown in Figure 2.

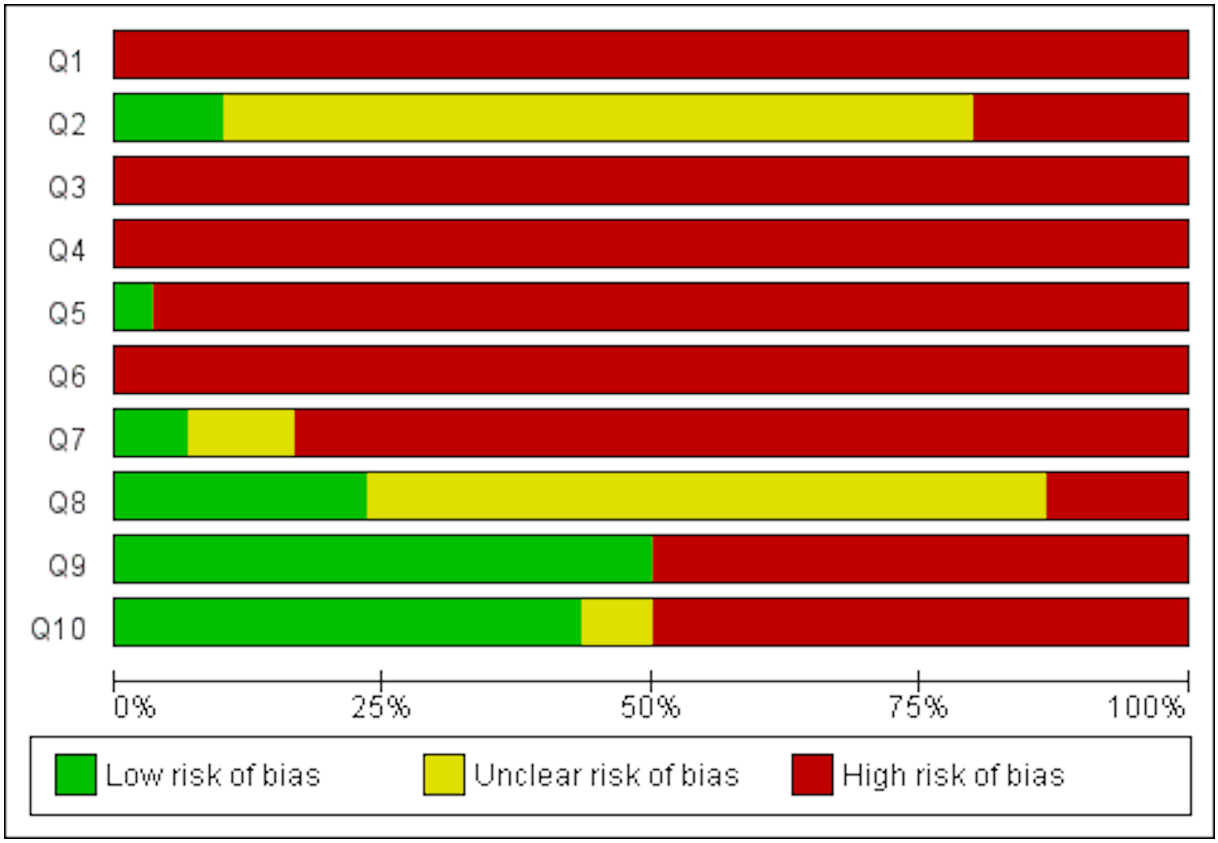

Figure 2. Results of the risk of bias for all studies included in the systematic review. The items in the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) Risk of Bias assessment (Q1–Q10) were scored with "yes" indicating low risk of bias, "no" indicating high risk of bias, or "unclear" indicating an unclear risk of bias. Q1–Q3 consider selection bias, Q4–Q5 consider performance bias, Q6–Q7 consider detection bias, Q8 considers attrition bias, Q9 considers reporting bias, and Q10 considers other biases. Q: Question. Q1: Was the allocation sequence adequately generated and applied?; Q2: Were the groups similar at baseline or were they adjusted for confounders in the analysis?; Q3: Was the allocation adequately concealed?; Q4: Were the animals randomly housed during the experiment?; Q5: Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?; Q6: Were animals selected at random for

outcome assessment?; Q7: Was the outcome assessor blinded?; Q8: Were incomplete outcome data adequately addressed?; Q9: Are reports of the study free of selective outcome reporting?; and Q10: Was the study apparently free of other problems that could result in high risk of bias?

Discussion

In order to meet a comprehensive interpretation of the evidence reported in this systematic review, in addition to the research outcomes, we conducted an analysis of the experimental models used in the selected studies to investigate the impact of animal peptides on skin wound healing. In our view, mapping these peptides and selecting well-designed animal models are critical for assessing the effectiveness of new molecules with healing potential. These aspects can contribute to clarify the potential biotechnological applicability of peptide-based strategies in regenerative medicine, an essential assumption to support clinical trials [45].

Relevance of animal models in studies on skin wound healing

Although pigs were used in only one study identified in the systematic review, this is the animal model whose skin is more similar to humans, which makes them an interesting model for preclinical studies on wound healing [46]. However, as these animals demand high husbandry costs and more restrictive ethical issues, their use has been increasingly limited. In contrast, mice and rats were the most used animal models, an aspect potentially associated with its greater availability, low cost and easy handling. In addition, mice, rats and humans exhibits the same stages of wound healing, with immuno-inflammatory and microstructural convergences mainly based on similar profiles of regulatory molecules (i.e., cytokines and growth factors) and composition of extracellular matrix (i.e., glycosaminoglycan's, collagen and non-collagen proteins) [47].

Rodents, especially mice and rats, are also often useful to investigate the effect of healing agents in pathological conditions such as diabetes [28,31,32], which was the associated disease most investigated in the studies reviewed. While streptozotocin was used to induce type I diabetes [31,32], type II diabetes was studied using *db/db* mice model [28]. Although diabetes develops from different physiopathological mechanisms in streptozotocin-induced and *db/db* animals, both models are valid to investigate the human disease. In this sense, induced-animals and diabetic humans share similar metabolic abnormalities, especially hyperglycemia, vasculopathy and neuropathy [48]. As these are disturbances associated with delayed wound healing in diabetes [49], chemically-induced and genetic models represent robust and realistic experimental constructs, which exhibits marked relevance and applicability in studies on healing products [28,31,32].

Relevance of wound models

The frequent use of rodents, excision wounds were consistently investigated in the studies reviewed. However, the number and size of the wounds were highly variable. Due to the complete skin removal, all phases of tissue repair are more pronounced in excisional than in incisional wounds [50]. Thus, excisional injuries are widely used in second intention healing models [22,26,31,40]. In these cases, the intense inflammatory process and the marked tissue remodeling favor the analysis of the effectiveness of healing products [26]. In addition to the type (first *vs.* second intention), the number of wounds exerts a relevant impact on the therapeutic outcome. Although most studies evaluated the healing potential of animal peptides on 1 or 2 wounds produced in each animal, 4 and 6 wounds were also reported. The main limitations of models based on multiple wounds are related to repeated biopsies on nearby

wounds [22,51]. As wound tissue collection creates additional damage to the skin, the acute inflammatory process is reactivated [51]. In this case, the upregulation of cytokine and growth factors might influence the adjacent wounds repair [52]. Thus, it would be ideal to investigate changes in only 1 wound per animal, to reduce the construct bias and its impact on the evidence. However, as models with 2 or more wounds are often required in time-dependent analysis of the healing process, the selection of these models should be carefully considered.

Regarding investigations on infected wounds, *S. aureus* was consistently used to induce wound infection. As *S. aureus* is an important human pathogen often associated to bacterial skin infections [53], preclinical models based on this bacteria are relevant and realistic. The emergence of multi-drug resistant microorganisms stimulates an important challenge in regenerative medicine: the development of more efficient products to treat infected wounds [14]. Efficient antimicrobial products are also relevant since the colonization of wounds by microorganisms amplifies inflammation and oxidative tissue damage, slowing or inhibiting the progression of the healing process [12,26,34]. Thus, studies on the treatment of infected wounds are urgent, especially considering that controlling infection is essential to reduce the risk of developing chronic wounds [54].

Relevance of therapeutic protocols

Although most studies used a diluted aqueous solution and applied the peptides topically, the number of applications and the treatment period was highly variable. The use of water, saline or sodium phosphate buffer as a vehicle indicated that animal peptides exhibit an interesting hydrophilic characteristic. These vehicles are relevant to avoid the development of cytotoxicity, which can occur with the use of organic solvents such as ethanol and dimethyl sulfoxide [55]. Unlike recommendations for different types of vehicle, there is no consensus on the dose and duration of treatment. Essentially, these aspects of dosimetry depend on the biological effect and the organic tolerability of each molecule. Thus, although the therapeutic effects are influenced by the dose and time of treatment, generalizations cannot be established for molecules with potentially different chemical and biological properties.

Effect of animal peptides on wound healing

Currently, identifying animal peptides with healing properties opens a new perspective for the treatment of skin wounds [16,32,34]. In general, reviewed studies indicate that peptides originating from mammals, amphibians, fishes, jellyfish, mollusk and insect exert beneficial effects in stimulating wound closure. However, peptides obtained from the fish species *Pardachirus marmoratus* [26] and *Oreochromis niloticus* [12,30] demonstrated positive effects only in infected wounds, suggesting that some peptides facilitate wound recovery by exerting antimicrobial effects and controlling opportunistic infections. This feature might be associated with the peptides' biochemical characteristics, since the peptides tilapia piscidin 3 (TP3) and tilapia piscidin 4 (TP4), both originating from *Oreochromis niloticus*, have similar amino acids sequence and share the same antibacterial action. Studies that evaluated mainly the antibacterial characteristics of the animal peptides showed a reduction of bacterial load on the wound area after treatment [12,26].

Animal peptides have been shown to act on the activation and proliferation of different cells involved in wound healing process. The increase in fibroblasts, myofibroblasts and endothelial cells potentiate the processes of dermal regeneration and wound closure, acting on the formation, contraction and nutrition of granulation tissue, respectively [25,27,36,41]. Several peptides were found to increase blood vessel density, in order to adequate nutrient and oxygen delivery to newly formed tissue [20,21,24,33]. There is evidence that animal peptides

may increase VEGF biosynthesis and stimulate neoangiogenesis, which is essential for a more efficient healing due to the influx of molecules required for the proper morphofunctional organization of the scar tissue [26,30,33,36]. TGF-β1 was additionally increased in response to animal peptides, which is a growth factor effective in stimulating cell proliferation, differentiation and migration; as well as colagenogenesis in the granulation tissue [13,27,36,39]. In addition, peptides obtained from mammals, amphibians, fishes and insect increased the recruitment of inflammatory cells, which contributes to the removal of damaged cells and matrix debris in the injured tissue and protects against local infections during the inflammatory phase, accelerating wound closure [13,21,23,26,27,34]. Several cells of the immune system are involved in wound healing, such as neutrophils, monocytes/macrophages, mast cells and lymphocytes [21]. Most studies have evaluated the effect of peptides on the recruitment of macrophages due to their critical roles in the healing process, coordinating complex processes of cell proliferation and extracellular matrix biosynthesis [12,13,21,26,27]. However, the peptide TP3, originating from the fish *Oreochromis niloticus* was effective in reducing the number of inflammatory cells in infected wounds, an effect related to the peptide's antimicrobial action in directly attenuating tissue bacterial load, reducing the antigenic load and immunological activation [12]. Although it remains poorly understood, these results help to clarify potential mechanisms of action of the peptides and their modulating action in the skin wound healing.

Some studies included in this review have evaluated the skin healing effect of animal peptides when comorbidities also occur, such as ischemia and diabetes. In the ischemic animal model, TP508 peptide from human thrombin significantly accelerated wound closure by stimulating anti-inflammatory processes and increasing tissue vascularization [22]. Diabetes was a condition widely studied, since the skin wound healing is often interrupted or delayed by abnormal glycation products and microvascular disturbances, contributing to the development of chronic wounds [56–59]. In addition, diabetic wounds often remain in the inflammatory stage for a long time, impairing the healing process due to the release of proinflammatory cytokines such as IL-6 and TNF-α [60]. Thus, studies evaluating the relevance of animal peptides on skin wound healing in diabetic animals are required, especially considering essential parameters such as immunological effectors, neoangiogenesis and wound closure to characterize the effect of the treatments. In diabetic models, most peptides identified significantly stimulated wound closure compared to untreated animals, increasing vascularization [31,32], colagenogenesis and dermal regeneration [32]. In addition, the human proinsulin C peptide reversed the increase of inflammatory cells in diabetic wounds, preventing an excessive inflammatory response and extensive secondary tissue damage, and consequently stimulating the rapid progression from the inflammatory to the proliferative phase [31]. These effects were associated with decreased pro-inflammatory cytokine production. Camel milk peptide increased activity of antioxidant enzymes such as SOD, CAT and GST; reducing the negative effects of excessive reactive oxygen species formation and lipid peroxidation [32]. However, a study testing CW49 peptide originating from the amphibian *Odorrana grahami* indicated a moderate effect on the healing process in a diabetic model [28]. In this study, wound closure was improved only in the early stages in the healing progression, when increased reepithelialization and dermal regeneration rates, blood vessel density, proangiogenic proteins and reduced recruitment of inflammatory cells and pro-inflammatory cytokines were reported.

Limitations

Systematic reviews are essential tools for summarizing evidence accurately and reliably, assisting risk assessment and providing evidence of the benefits of health-related interventions [61]. However, the methodological quality of the studies included in this review was

predominantly classified as high risk or unclear risk of bias, indicating that most features needed for a bias study evaluation were not sufficiently reported. Incomplete characterization of animal models, peptide acquisition and characterization, treatment protocols, outcome measures and mechanisms involved in the healing process all contributed to the increased risk of bias. Along with these limitations, results were presented only as graphics in most studies, which made it difficult to assess the absolute values related to the wound area. We hope that our critical analysis helps accelerating preclinical research and reducing methodological bias by improving experimental control and accuracy of research reports.

Conclusion

In general, we identified that the evidence on the healing potential of animal peptides is mainly based on valid and realistic preclinical models that share similar tissue repair phases with those observed in humans. From studies using these models, we identified that animal peptides are potentially effective in accelerating the skin wound healing. For most of the identified peptides, the beneficial effect is mainly associated with cell proliferation stimulation, neoangiogenesis, colagenogenesis, reepithelization and wound contraction. However, the healing property of a small group of tilapia-derived peptides (TP3 and TP4) is potentially related to the antibacterial effects of these molecules. Despite the beneficial healing effects, the risk of bias and methodological divergences observed in some studies make the current evidence limited to the experimental contexts applied to the animal models analyzed. Considering that research papers on animal peptides promoting wound healing are relatively recent, there is a growing need to increase the number of investigations and improve the experimental protocols and research reports. We hope that our critical analysis helps accelerating preclinical research and reducing methodological bias by improving experimental control and accuracy of the research reports in this area.

Conflicts of interest

No competing financial interests exist.

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Supplementary material

S1 Table. Complete search strategy with search filters and number of research records recovered in the PubMed-Medline, Scopus and Web of Science databases. *: In PubMed-Medline database, standardized animal filters were obtained in "Hooijmans CR, Tillema A, Leenaars M, Ritskes-Hoitinga M. Enhancing search efficiency by means of a search filter for finding all studies on animal experimentation in PubMed. Laboratory Animals 2010;44:170-175."

S2 Table. Studies excluded during the process of eligiblity.

S3 Table. General characteristics of the preclinical models used in all studies investigating the relevance of animal peptides in the treatment of skin wounds. ♂: Male, ♀: Female, ?: Not reported or unclear, wk: Weeks.

S4 Table. General characteristics of skin wounds used in preclinical models investigating the relevance of animal peptides as healing agents. ?: Not reported or unclear, *S. aureus*: *Staphylococcus aureus*, *E. coli: Escherichia coli*, D: Diameter, CFU: Colony forming unit.

S5 Table. Description of the main characteristics related to peptides included in the systematic review on peptides of animal origin applied in the treatment of skin wounds.

S6 Table. Treatment protocols used in all studies investigating the relevance of animal peptides in the treatment of skin wounds. ?: Not reported or unclear, SAL: Saline solution, PBS: Phosphate buffered saline solution, DPBS: Dulbecco's phosphate-buffered saline, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously.

S7 Table. PRISMA 2009 Checklist. *From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097.

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S1 Table. Complete search strategy with search filters and number of research records recovered in the PubMed-Medline, Scopus and Web of Science databases.

PubMed-MEDLINE - Search Filters	Retrieved records
#1 Peptides ("peptides"[TIAB] OR "antioxidant peptides"[TIAB] OR "antimicrobial peptides"[TIAB] OR "angiogenic peptides"[TIAB] OR "animal peptides"[TIAB] OR "natural peptides"[TIAB] OR "bioactive peptides"[TIAB] OR "biological peptides"[TIAB] OR "isolated peptides"[TIAB] OR "extracted peptides"[TIAB])	**228261**
#2 Wound Healing ("wound healing"[MeSH Terms] OR "regeneration"[MeSH Terms] OR "regeneration"[TIAB] OR "wound healing"[TIAB] OR "skin repair"[TIAB] OR "cutaneous repair"[TIAB] OR "skin healing"[TIAB] OR "cutaneous healing"[TIAB])	**337601**
#3 Skin ("skin"[MeSH Terms] OR "dermis"[MeSH Terms] OR "epidermis"[MeSH Terms] OR "subcutaneous tissue"[MeSH Terms] OR "granulation tissue"[MeSH Terms] OR "keratinocytes"[MeSH Terms] OR "fibroblasts"[MeSH Terms] OR "integumentary system"[MeSH Terms] OR "skin"[TIAB] OR "dermis"[TIAB] OR "epidermis"[TIAB] OR "subcutaneous tissue"[TIAB] OR "hypodermis"[TIAB] OR "granulation tissue"[TIAB] OR "keratinocytes"[TIAB] OR "fibroblasts"[TIAB] OR "integumentary system"[TIAB] OR "skin injuries"[TIAB] OR "skin fibrosis"[TIAB] OR "skin scars"[TIAB])	**955157**
#4 First animal filter* ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms])	**6707109**
#5 Second animal filter* ("animals"[TIAB] OR "animal"[TIAB] OR "mice"[TIAB] OR "mus"[TIAB] OR "mouse"[TIAB] OR "murine"[TIAB] OR "woodmouse"[TIAB] OR "rats"[TIAB] OR "rat"[TIAB] OR "murinae"[TIAB] OR "muridae"[TIAB] OR "cottonrat"[TIAB] OR "cottonrats"[TIAB] OR "hamster"[TIAB] OR "hamsters"[TIAB] OR "cricetinae"[TIAB] OR "rodentia"[TIAB] OR "rodent"[TIAB] OR "rodents"[TIAB] OR "pigs"[TIAB] OR "pig"[TIAB] OR "swine"[TIAB] OR "swines"[TIAB] OR "piglets"[TIAB] OR "piglet"[TIAB] OR "boar"[TIAB] OR "boars"[TIAB] OR "sus scrofa"[TIAB] OR "ferrets"[TIAB] OR "ferret"[TIAB] OR "polecat"[TIAB] OR "polecats"[TIAB] OR "mustela putorius"[TIAB] OR "guinea pigs"[TIAB] OR "guinea pig"[TIAB] OR "cavia"[TIAB] OR "callithrix"[TIAB] OR "marmoset"[TIAB] OR "marmosets"[TIAB] OR "cebuella"[TIAB] OR "hapale"[TIAB] OR "octodon"[TIAB] OR "chinchilla"[TIAB] OR "chinchillas"[TIAB] OR "gerbillinae"[TIAB] OR "gerbil"[TIAB] OR "gerbils"[TIAB] OR "jird"[TIAB] OR "jirds"[TIAB] OR "merione"[TIAB] OR "meriones"[TIAB] OR "rabbits"[TIAB] OR "rabbit"[TIAB] OR "hares"[TIAB] OR "hare"[TIAB] OR "diptera"[TIAB] OR "flies"[TIAB] OR "fly"[TIAB] OR "dipteral"[TIAB] OR "drosphila"[TIAB] OR "drosophilidae"[TIAB] OR "cats"[TIAB] OR "cat"[TIAB] OR "carus"[TIAB] OR "felis"[TIAB] OR "nematoda"[TIAB] OR "nematode"[TIAB] OR "nematoda"[TIAB] OR "nematode"[TIAB] OR "nematodes"[TIAB] OR "sipunculida"[TIAB] OR "dogs"[TIAB] OR "dog"[TIAB] OR "canine"[TIAB] OR "canines"[TIAB] OR "canis"[TIAB] OR "sheep"[TIAB] OR "sheeps"[TIAB] OR "mouflon"[TIAB] OR "mouflons"[TIAB] OR "ovis"[TIAB] OR "goats"[TIAB] OR "goat"[TIAB] OR "capra"[TIAB]	**4553894**

OR "capras"[TIAB] OR "rupicapra"[TIAB] OR "chamois"[TIAB] OR "haplorhini"[TIAB] OR "monkey"[TIAB] OR "monkeys"[TIAB] OR "anthropoidea"[TIAB] OR "anthropoids"[TIAB] OR "saguinus"[TIAB] OR "tamarin"[TIAB] OR "tamarins"[TIAB] OR "leontopithecus"[TIAB] OR "hominidae"[TIAB] OR "ape"[TIAB] OR "apes"[TIAB] OR "pan"[TIAB] OR "paniscus"[TIAB] OR "pan paniscus"[TIAB] OR "bonobo"[TIAB] OR "bonobos"[TIAB] OR "troglodytes"[TIAB] OR "pan troglodytes"[TIAB] OR "gibbon"[TIAB] OR "gibbons"[TIAB] OR "siamang"[TIAB] OR "siamangs"[TIAB] OR "nomascus"[TIAB] OR "symphalangus"[TIAB] OR "chimpanzee"[TIAB] OR "chimpanzees"[TIAB] OR "prosimians"[TIAB] OR "bush baby"[TIAB] OR "prosimian"[TIAB] OR "bush babies"[TIAB] OR "galagos"[TIAB] OR "galago"[TIAB] OR "pongidae"[TIAB] OR "gorilla"[TIAB] OR "gorillas"[TIAB] OR "pongo"[TIAB] OR "pygmaeus"[TIAB] OR "pongo pygmaeus"[TIAB] OR "orangutans"[TIAB] OR "pygmaeus"[TIAB] OR "lemur"[TIAB] OR "lemurs"[TIAB] OR "lemuridae"[TIAB] OR "horse"[TIAB] OR "horses"[TIAB] OR "pongo"[TIAB] OR "equus"[TIAB] OR "cow"[TIAB] OR "calf"[TIAB] OR "bull"[TIAB] OR "chicken"[TIAB] OR "chickens"[TIAB] OR "gallus"[TIAB] OR "quail"[TIAB] OR "bird"[TIAB] OR "birds"[TIAB] OR "quails"[TIAB] OR "poultry"[TIAB] OR "poultries"[TIAB] OR "fowl"[TIAB] OR "fowls"[TIAB] OR "reptile"[TIAB] OR "reptilia"[TIAB] OR "reptiles"[TIAB] OR "snakes"[TIAB] OR "snake"[TIAB] OR "lizard"[TIAB] OR "lizards"[TIAB] OR "alligator"[TIAB] OR "alligators"[TIAB] OR crocodile[TIAB] OR "crocodiles"[TIAB] OR "turtle"[TIAB] OR "turtles"[TIAB] OR "amphibian"[TIAB])	
#6 Third animal filter* ("amphibians"[TIAB] OR "amphibia"[TIAB] OR "frog"[TIAB] OR "frogs"[TIAB] OR "bombina"[TIAB] OR "salientia"[TIAB] OR "toad"[TIAB] OR "toads"[TIAB] OR "epidalea calamita"[TIAB] OR "salamander"[TIAB] OR "salamanders"[TIAB] OR "eel"[TIAB] OR "eels"[TIAB] OR "fish"[TIAB] OR "fishes"[TIAB] OR "pisces"[TIAB] OR "catfish"[TIAB] OR "catfishes"[TIAB] OR "siluriformes"[TIAB] OR "arius"[TIAB] OR "heteropneustes"[TIAB] OR "sheatfish"[TIAB] OR "perch"[TIAB] OR "perches"[TIAB] OR "percidae"[TIAB] OR "perca"[TIAB] OR "trout"[TIAB] OR "trouts"[TIAB] OR "char"[TIAB] OR "chars"[TIAB] OR "salvelinus"[TIAB] OR "fathead minnow"[TIAB] OR "minnow"[TIAB] OR "cyprinidae"[TIAB] OR "carps"[TIAB] OR "carp"[TIAB] OR "zebrafish"[TIAB] OR "zebrafishes"[TIAB] OR "goldfish"[TIAB] OR "goldfishes"[TIAB] OR "guppy"[TIAB] OR "guppies"[TIAB] OR "chub"[TIAB] OR "chubs"[TIAB] OR "tinca"[TIAB] OR "barbels"[TIAB] OR "barbus"[TIAB] OR "pimephales"[TIAB] OR "promelas"[TIAB] OR "poecilia reticulata"[TIAB] OR "mullet"[TIAB] OR "mullets"[TIAB] OR "seahorse"[TIAB] OR "seahorses"[TIAB] OR "mugil curema"[TIAB] OR "atlantic cod"[TIAB] OR "shark"[TIAB] OR "sharks"[TIAB] OR "catshark"[TIAB] OR "anguilla"[TIAB] OR "salmonid"[TIAB] OR "salmonids"[TIAB] OR "whitefish"[TIAB] OR "whitefishes"[TIAB] OR "salmon"[TIAB] OR "salmons"[TIAB] OR "sole"[TIAB] OR "solea"[TIAB] OR "sea lamprey"[TIAB] OR "lamprey"[TIAB] OR "lampreys"[TIAB] OR "pumpkinseed"[TIAB] OR "sunfish"[TIAB] OR "sunfishes"[TIAB] OR "tilapia"[TIAB] OR "tilapias"[TIAB] OR "turbot"[TIAB] OR "turbots"[TIAB] OR "flatfish"[TIAB] OR "flatfishes"[TIAB] OR "sciuridae"[TIAB] OR "squirrel"[TIAB] OR "squirrels"[TIAB] OR "chipmunk"[TIAB] OR "chipmunks"[TIAB] OR "suslik"[TIAB] OR "susliks"[TIAB] OR "vole"[TIAB] OR "voles"[TIAB] OR "lemming"[TIAB] OR "lemmings"[TIAB] OR "muskrat"[TIAB] OR "muskrats"[TIAB] OR "lemmus"[TIAB] OR "otter"[TIAB] OR "otters"[TIAB] OR "marten"[TIAB] OR "martens"[TIAB] OR "martes"[TIAB] OR "weasel"[TIAB] OR "badger"[TIAB] OR "badgers"[TIAB] OR "ermine"[TIAB] OR "mink"[TIAB] OR "minks"[TIAB] OR "sable"[TIAB] OR "sables"[TIAB] OR "gulo"[TIAB] OR "gulos"[TIAB] OR "wolverine"[TIAB] OR "wolverines"[TIAB] OR "minks"[TIAB] OR "mustela"[TIAB] OR "llama"[TIAB] OR "llamas"[TIAB] OR "alpaca"[TIAB] OR "alpacas"[TIAB] OR "camelid"[TIAB] OR "camelids"[TIAB] OR "guanaco"[TIAB] OR "guanacos"[TIAB] OR "chiroptera"[TIAB] OR "chiropteras"[TIAB] OR "bat"[TIAB] OR "bats"[TIAB] OR "fox"[TIAB] OR "foxes"[TIAB] OR "iguana"[TIAB] OR "iguanas"[TIAB] OR "xenopus laevis"[TIAB] OR "parakeet"[TIAB] OR "parakeets"[TIAB] OR "parrot"[TIAB] OR "parrots"[TIAB] OR "donkey"[TIAB] OR "donkeys"[TIAB] OR "mule"[TIAB] OR "mules"[TIAB] OR "zebra"[TIAB] OR "zebras"[TIAB] OR "shrew"[TIAB] OR "shrews"[TIAB] OR "bison"[TIAB] OR "bisons"[TIAB] OR "buffalo"[TIAB] OR "buffaloes"[TIAB] OR "deer"[TIAB] OR "deers"[TIAB] OR "bear"[TIAB] OR "bears"[TIAB] OR "panda"[TIAB] OR "pandas"[TIAB] OR "wild hog"[TIAB] OR "wild boar"[TIAB] OR	**499752**

"fitchew"[TIAB] OR "fitch"[TIAB] OR "beaver"[TIAB] OR "beavers"[TIAB] OR "jerboa"[TIAB] OR "jerboas"[TIAB] OR "capybara"[TIAB] OR "capybaras"[TIAB])	
Combined search: (((((#6) OR (#5)) OR (#4)) AND (#3)) AND (#2)) AND (#1)	**422**

SCOPUS - Search Filters	**Retrieved records**
#1 Peptides (TITLE-ABS-KEY("peptides") OR TITLE-ABS-KEY("antioxidant peptides") OR TITLE-ABS-KEY ("antimicrobial peptides") OR TITLE-ABS-KEY("angiogenic peptides") OR TITLE-ABS-KEY ("animal peptides") OR TITLE-ABS-KEY("natural peptides") OR TITLE-ABS-KEY("bioactive peptides") OR TITLE-ABS-KEY("biological peptides") OR TITLE-ABS-KEY("isolated peptides") OR TITLE-ABS-KEY("extracted peptides"))	**938519**
#2 Wound Healing (TITLE-ABS-KEY("wound healing") OR TITLE-ABS-KEY("regeneration") OR TITLE-ABS-KEY("skin repair") OR TITLE-ABS-KEY("cutaneous repair") OR TITLE-ABS-KEY("skin healing") OR TITLE-ABS-KEY("cutaneous healing"))	**482869**
#3 Skin (TITLE-ABS-KEY("skin") OR TITLE-ABS-KEY("dermis") OR TITLE-ABS-KEY ("epidermis") OR TITLE-ABS-KEY("subcutaneous tissue") OR TITLE-ABS-KEY("hypodermis") OR TITLE-ABS-KEY("granulation tissue") OR TITLE-ABS-KEY("keratinocytes") OR TITLE-ABS-KEY("fibroblasts") OR TITLE-ABS-KEY ("integumentary system") OR TITLE-ABS-KEY("skin injuries") OR TITLE-ABS-KEY("skin fibrosis") OR TITLE-ABS-KEY("skin scars"))	**1599969**
Combined search: #3 AND #2 AND #1	**3908**
Search limits (Keyword): Animal Experiment and English	**926**

Web of Science - Search Filters	**Retrieved records**
#1 Peptides TS=("peptides" OR "antioxidant peptides" OR "antimicrobial peptides" OR "angiogenic peptides" OR "animal peptides" OR "natural peptides" OR "bioactive peptides" OR "biological peptides" OR "isolated peptides" OR "extracted peptides")	**295819**
#2 Wound Healing TS=("wound healing" OR "regeneration" OR "skin repair" OR "cutaneous repair" OR "skin healing" OR "cutaneous healing")	**311314**
#3 Skin TS=("skin" OR "dermis" OR "epidermis" OR "subcutaneous tissue" OR "hypodermis" OR "granulation tissue" OR "keratinocytes" OR "fibroblasts" OR "integumentary system" OR "skin injuries" OR "skin fibrosis" OR "skin scars")	**788902**
#4 First animal filter* TS=("animal experimentation" OR "models, animal" OR "invertebrates" OR "animals" OR "animal population groups" OR "chordata" OR "chordata, nonvertebrate" OR "vertebrates" OR "amphibians" OR "birds" OR "fishes" OR "reptiles" OR "mammals" OR "primates" OR "artiodactyla" OR "carnivora" OR "cetacea" OR "chiroptera" OR "elephants" OR "hyraxes" OR "insectivora" OR "lagomorpha" OR "marsupialia" OR "monotremata" OR "perissodactyla" OR "rodentia" OR "scandentia" OR "sirenia" OR "xenarthra" OR "haplorhini" OR "strepsirhini" OR "platyrrhini" OR "tarsii" OR "catarrhini" OR "cercopithecidae" OR "hylobatidae" OR "hominidae" OR "gorilla gorilla" OR "pan paniscus" OR "pan troglodytes" OR "pongo pygmaeus")	**1075322**
#5 Second animal filter* TS=("animals" OR "animal" OR "mice" OR "mus" OR "mouse" OR "murine" OR "woodmouse" OR "rats" OR "rat" OR "murinae" OR "muridae" OR "cottonrat" OR "cottonrats" OR "hamster" OR "hamsters" OR "cricetinae" OR "rodentia" OR "rodent" OR "rodents" OR "pigs" OR "pig" OR "swine" OR "swines" OR "piglets" OR "piglet" OR "boar" OR "boars" OR "sus scrofa" OR "ferrets" OR "ferret" OR "polecat" OR "polecats" OR "mustela putorius" OR "guinea pigs" OR "guinea pig" OR "cavia" OR "callithrix" OR "marmoset" OR "marmosets"	**5837275**

OR "cebuella" OR "hapale" OR "octodon" OR "chinchilla" OR "chinchillas" OR "gerbillinae" OR "gerbil" OR "gerbils" OR "jird" OR "jirds" OR "merione" OR "meriones" OR "rabbits" OR "rabbit" OR "hares" OR "hare" OR "diptera" OR "flies" OR "fly" OR "dipteral" OR "drosphila" OR "drosophilidae" OR "cats" OR "cat" OR "carus" OR "felis" OR "nematoda" OR "nematode" OR "nematoda" OR "nematode" OR "nematodes" OR "sipunculida" OR "dogs" OR "dog" OR "canine" OR "canines" OR "canis" OR "sheep" OR "sheeps" OR "mouflon" OR "mouflons" OR "ovis" OR "goats" OR "goat" OR "capra" OR "capras" OR "rupicapra" OR "chamois" OR "haplorhini" OR "monkey" OR "monkeys" OR "anthropoidea" OR "anthropoids" OR "saguinus" OR "tamarin" OR "tamarins" OR "leontopithecus" OR "hominidae" OR "ape" OR "apes" OR "pan" OR "paniscus" OR "pan paniscus" OR "bonobo" OR "bonobos" OR "troglodytes" OR "pan troglodytes" OR "gibbon" OR "gibbons" OR "siamang" OR "siamangs" OR "nomascus" OR "symphalangus" OR "chimpanzee" OR "chimpanzees" OR "prosimians" OR "bush baby" OR "prosimian" OR "bush babies" OR "galagos" OR "galago" OR "pongidae" OR "gorilla" OR "gorillas" OR "pongo" OR "pygmaeus" OR "pongo pygmaeus" OR "orangutans" OR "pygmaeus" OR "lemur" OR "lemurs" OR "lemuridae" OR "horse" OR "horses" OR "pongo" OR "equus" OR "cow" OR "calf" OR "bull" OR "chicken" OR "chickens" OR "gallus" OR "quail" OR "bird" OR "birds" OR "quails" OR "poultry" OR "poultries" OR "fowl" OR "fowls" OR "reptile" OR "reptilia" OR "reptiles" OR "snakes" OR "snake" OR "lizard" OR "lizards" OR "alligator" OR "alligators" OR "crocodile" OR "crocodiles" OR "turtle" OR "turtles" OR "amphibian")	
#6 Third animal filter* TS=("amphibians" OR "amphibia" OR "frog" OR "frogs" OR "bombina" OR "salientia" OR "toad" OR "toads" OR "epidalea calamita" OR "salamander" OR "salamanders" OR "eel" OR "eels" OR "fish" OR "fishes" OR "pisces" OR "catfish" OR "catfishes" OR "siluriformes" OR "arius" OR "heteropneustes" OR "sheatfish" OR "perch" OR "perches" OR "percidae" OR "perca" OR "trout" OR "trouts" OR "char" OR "chars" OR "salvelinus" OR "fathead minnow" OR "minnow" OR "cyprinidae" OR "carps" OR "carp" OR "zebrafish" OR "zebrafishes" OR "goldfish" OR "goldfishes" OR "guppy" OR "guppies" OR "chub" OR "chubs" OR "tinca" OR "barbels" OR "barbus" OR "pimephales" OR "promelas" OR "poecilia reticulata" OR "mullet" OR "mullets" OR "seahorse" OR "seahorses" OR "mugil curema" OR "atlantic cod" OR "shark" OR "sharks" OR "catshark" OR "anguilla" OR "salmonid" OR "salmonids" OR "whitefish" OR "whitefishes" OR "salmon" OR "salmons" OR "sole" OR "solea" OR "sea lamprey" OR "lamprey" OR "lampreys" OR "pumpkinseed" OR "sunfish" OR "sunfishes" OR "tilapia" OR "tilapias" OR "turbot" OR "turbots" OR "flatfish" OR "flatfishes" OR "sciuridae" OR "squirrel" OR "squirrels" OR "chipmunk" OR "chipmunks" OR "suslik" OR "susliks" OR "vole" OR "voles" OR "lemming" OR "lemmings" OR "muskrat" OR "muskrats" OR "lemmus" OR "otter" OR "otters" OR "marten" OR "martens" OR "martes" OR "weasel" OR "badger" OR "badgers" OR "ermine" OR "mink" OR "minks" OR "sable" OR "sables" OR "gulo" OR "gulos" OR "wolverine" OR "wolverines" OR "minks" OR "mustela" OR "llama" OR "llamas" OR "alpaca" OR "alpacas" OR "camelid" OR "camelids" OR "guanaco" OR "guanacos" OR "chiroptera" OR "chiropteras" OR "bat" OR "bats" OR "fox" OR "foxes" OR "iguana" OR "iguanas" OR "xenopus laevis" OR "parakeet" OR "parakeets" OR "parrot" OR "parrots" OR "donkey" OR "donkeys" OR "mule" OR "mules" OR "zebra" OR "zebras" OR "shrew" OR "shrews" OR "bison" OR "bisons" OR "buffalo" OR "buffaloes" OR "deer" OR "deers" OR "bear" OR "bears" OR "panda" OR "pandas" OR "wild hog" OR "wild boar" OR "fitchew" OR "fitch" OR "beaver" OR "beavers" OR "jerboa" OR "jerboas" OR "capybara" OR "capybaras")	**1034243**
Combined search: (((((#6) OR (#5)) OR (#4)) AND (#3)) AND (#2)) AND (#1) **Search limits (Keyword):** English	**386**

*: In PubMed-Medline and Web of Science databases, standardized animal filters were obtained in "Hooijmans CR, Tillema A, Leenaars M, Ritskes-Hoitinga M. Enhancing search efficiency by means of a search filter for finding all studies on animal experimentation in PubMed. Laboratory Animals 2010;44:170-175.

S2 Table. Studies excluded during the process of eligibility.

Exclusion criteria	Studies	Number
Association with other compounds	Anti-infective biomaterials with surface-decorated tachyplesin I A bio-inspired, microchanneled hydrogel with controlled spacing of cell adhesion ligands regulates 3D spatial organization of cells and tissue A hybrid system of hydrogel/frog egg-like microspheres accelerates wound healing via sustained delivery of RCSPs A Novel Functionalization of Bioactive Antimicrobial Peptide onto the Nano-CeO2/Reduced Graphene Oxide Cluster Type Biocomposite Wound Dressings for Diabetic Wound Care Management: In Vitro and In Vivo Evaluations A peptide-morpholino oligomer conjugate targeting Staphylococcus aureus gyrA mRNA improves healing in an infected mouse cutaneous wound mode A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices Accelerated healing of excisional skin wounds by PL 14736 in alloxan-hyperglycemic rats Acceleration of diabetic wound healing by an angiopoietin peptide mimetic An identified antioxidant peptide obtained from ostrich (*Struthio camelus*) egg white protein hydrolysate shows wound healing properties Analysis of healing effect of alginate sulfate hydrogel dressing containing antimicrobial peptide on wound infection caused by methicillin-resistant Staphylococcus aureus Angiogenic Heparin-Mimetic Peptide Nanofiber Gel Improves Regenerative Healing of Acute Wounds Angiogenic Peptide Nanofibers Improve Wound Healing in STZ-Induced Diabetic Rats Antimicrobial peptide-gold nanoscale therapeutic formulation with high skin regenerative potential Assessment of antimicrobial and wound healing effects of Brevinin-2Ta against the bacterium Klebsiella pneumoniae in dermally-wounded rats Atrial natriuretic peptide accelerates human endothelial progenitor cell–stimulated cutaneous wound healing and angiogenesis Bioactive peptide amphiphile nanofiber gels enhance burn wound healing Biofunctions of antimicrobial peptide-conjugated alginate/hyaluronic acid/collagen wound dressings promote wound healing of a mixed-bacteria-infected wound Biomacromolecule immobilization: Grafting of fish-scale collagen peptides onto aminolyzed P(3HB-co-4HB) scaffolds as a potential wound dressing Biotinylated GHK peptide incorporated collagenous matrix: A novel biomaterial for dermal wound healing in rats Body protective compound-157 enhances alkali-burn wound healing in vivo and promotes proliferation, migration, and angiogenesis in vitro	77

Carboxymethyl chitosan nanoparticles loaded with bioactive peptide OH-CATH30 benefit nonscar wound healing

Chitosan hydrogel encapsulated with LL-37 peptide promotes deep tissue injury healing in a mouse model

Chitosan hydrogel in combination with marine peptides from tilapia for burns healing

Co-assembled supramolecular hydrogels of cell adhesive peptide and alginate for rapid hemostasis and efficacious wound healing

Connexin43 carboxyl-terminal peptides reduce scar progenitor and promote regenerative healing following skin wounding

Design of antimicrobial peptides conjugated biodegradable citric acid derived hydrogels for wound healing

Development of alginate wound dressings linked with hybrid peptides derived from laminin and elastin

Development of angiotensin (1-7) as an agent to accelerate dermal repair

Diabetic wound regeneration using heparin-mimetic peptide amphiphile gel in db/db mice

Diabetic wound regeneration using peptide-modified hydrogels to target re-epithelialization

Effect of fibrin-binding synthetic oligopeptide on the healing of full-thickness skin wounds in streptozotocin-induced diabetic rats

Effect of synthetic peptide thrombin receptor agonist encapsulated in microparticles based on lactic and glycolic acid copolymer on healing of experimental skin wounds in mice

Electrospinning of In situ crosslinked recombinant human collagen peptide/chitosan nanofibers for wound healing

Electrospun poly(l-lactide)/zein nanofiber mats loaded with *Rana chensinensis* skin peptides for wound dressing.

Encapsulation of collagen mimetic peptide-tethered vancomycin liposomes in collagen-based scaffolds for infection control in wounds

Evaluation of Small Molecular Polypeptides from the Mantle of *Pinctada Martensii* on Promoting Skin Wound Healing in Mice

Exendin-4 in combination with adipose-derived stem cells promotes angiogenesis and improves diabetic wound healing

Fabrication of KR-12 peptide-containing hyaluronic acid immobilized fibrous eggshell membrane effectively kills multi-drug-resistant bacteria, promotes angiogenesis and accelerates re-epithelialization.

Fragments of Nle3-angiotensin(1-7) accelerate healing in dermal models

Heparin mimetic peptide nanofiber gel promotes regeneration of full thickness burn injury

Histological evaluation of the effects of angiotensin peptides on wound repair in diabetic mice

Identification and characterization of novel matrix-derived bioactive peptides: A role for collagenase from santyl1 ointment in post-debridement wound healing?

Immobilized thrombin receptor agonist peptide accelerates wound healing in mice

In situ gel-forming AP-57 peptide delivery system for cutaneous wound healing

In vivo effects of tailored laminin-332 α3 conjugated scaffolds enhances wound healing: A histomorphometric analysis

Laminin heparin-binding peptides bind to several growth factors and enhance diabetic wound healing

Laminin peptide-conjugated chitosan membrane: Application for keratinocyte delivery in wounded skin

Light-Activated Peptide-Based Materials for Sutureless Wound Closure

LL37 loaded nanostructured lipid carriers (NLC): A new strategy for the topical treatment of chronic wounds

Matrix- and plasma-derived peptides promote tissue-specific injury responses and wound healing in diabetic swine

Modeling Glyco-Collagen Conjugates Using a Host-Guest Strategy To Alter Phenotypic Cell Migration and in Vivo Wound Healing

Multidomain Peptide Hydrogel Accelerates Healing of Full-Thickness Wounds in Diabetic Mice

Oligoarginine mediated collagen/chitosan gel composite for cutaneous wound healing

Pentadecapeptide BPC 157 cream improves burn-wound healing and attenuates burn-gastric lesions in mice

Peptide-Modified Chitosan Hydrogels Accelerate Skin Wound Healing by Promoting Fibroblast Proliferation, Migration, and Secretion

Peptide-modified chitosan hydrogels promote skin wound healing by enhancing wound angiogenesis and inhibiting inflammation

Pexiganan-incorporated collagen matrices for infected wound-healing processes in rat

Pigment epithelium-derived factor short peptides facilitate full-thickness cutaneous wound healing by promoting epithelial basal cell and hair follicle stem cell proliferation

PLGA nanoparticles loaded with host defense peptide LL37 promote wound healing

Polyvinyl alcohol nanofiber formulation of the designer antimicrobial peptide APO sterilizes *Acinetobacter baumannii*-infected skin wounds in mice

Potential therapeutic efficacy of a bactericidal-immunomodulatory fusion peptide against methicillin-resistant Staphylococcus aureus skin infection

Simultaneous dual syringe electrospinning system using benign solvent to fabricate nanofibrous P(3HB-co-4HB)/collagen peptides construct as potential leave-on wound dressing

Skin Regeneration with a Scaffold of Predefined Shape and Bioactive Peptide Hydrogels

Skin Regeneration with Self-Assembled Peptide Hydrogels Conjugated with Substance P in a Diabetic Rat Model

Sponges of Carboxymethyl Chitosan Grafted with Collagen Peptides for Wound Healing.

Stromal Cell-Derived Growth Factor-1 Alpha-Elastin Like Peptide Fusion Protein Promotes Cell Migration and Revascularization of Experimental Wounds in Diabetic Mice

	Substance P accelerates wound healing in type 2 diabetic mice through endothelial progenitor cell mobilization and Yes-associated protein activation	
	Sustained-release adrenomedullin ointment accelerates wound healing of pressure ulcers	
	Synthesis and assessment of a novel peptide conjugate to deliver phenytoin for wound repair	
	TGF-β1 latency associated peptide promotes remodeling of healing cutaneous wounds in the rat	
	The development and characterization of SDF1α-elastin-like-peptide nanoparticles for wound healing	
	The effect of a laminin-5-derived peptide coated onto chitin microfibers on re-epithelialization in early-stage wound healing	
	The effects of Antimicrobial Peptides and Hyaluronic Acid compound mask on wound healing after ablative fractional Carbon Dioxide laser resurfacing	
	The wound healing effects of the Tilapia collagen peptide mixture TY001 in streptozotocin diabetic mice	
	Therapeutic effects of a recombinant human collagen peptide bioscaffold with human adipose-derived stem cells on impaired wound healing after radiotherapy	
	Thermosensitive biomimetic polyisocyanopeptide hydrogels may facilitate wound repair	
	Ultrashort peptide nanofibrous hydrogels for the acceleration of healing of burn wounds	
Peptides of unreported origin	Angiogenic laminin-derived peptides stimulate wound healing	13
	Antimicrobial endotoxin-neutralizing peptides promote keratinocyte migration via P2X7 receptor activation and accelerate wound healing in vivo	
	Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes	
	Efficacy of the quorum sensing inhibitor FS10 alone and in combination with tigecycline in an animal model of staphylococcal infected wound	
	Egg White peptide KPHAEVVLR promotes skin fibroblasts migration and mice skin wound healing by stimulating cell membrane Hsp90 alpha secretion	
	Enhancement by PL 14736 of granulation and collagen organization in healing wounds and the potential role of egr-1 expression	
	Exendin-4, a glucagon-like peptide-1 analogue, accelerates diabetic wound healing	
	Glu-Trp-ONa or its acylated analogue (R-Glu-Trp-ONa) administration enhances the wound healing in the model of chronic skin wounds in rabbits	
	Highly selective end-tagged antimicrobial peptides derived from PRELP	
	Innate defense regulator peptide 1018 in wound healing and wound infection	
	The effects of brain natriuretic peptide on scar formation in incisional rat wounds	
	The pro-healing effect of exendin-4 on wounds produced by abrasion in normoglycemic mice	
	Variants of self-assembling peptide, KLD-12 that show both rapid fracture healing and antimicrobial properties	

Non-animal peptides	A peptide inhibitor of c-Jun promotes wound healing in a mouse full-thickness burn model A RHAMM mimetic peptide blocks hyaluronan signaling and reduces inflammation and fibrogenesis in excisional skin wounds A small peptide with potential ability to promote wound healing Discovery and characterization of a high-affinity small peptide ligand, H1, targeting FGFR2IIIc for Skin Wound Healing Effect of tripeptide-copper complexes on the process of skin wound healing and on cultured fibroblasts. Efficacy of the designer antimicrobial peptide SHAP1 in wound healing and wound infection. Myxinidin2 and myxinidin3 suppress inflammatory responses through STAT3 and MAPKs to promote wound healing Topical application of Sadat-Habdan mesenchymal stimulating peptide (SHMSP) accelerates wound healing in diabetic rabbits Wound healing acceleration of a novel transforming growth factor-β inducer, SEK-1005	9
Sutured wounds	Effect of NorLeu3-A(1-7) on scar formation over time after full-thickness incision injury in the rat Exogenously-administered Leptin increases early incisional wound angiogenesis in an experimental animal model Impact of single-dose application of TGF-β, copper peptide, stanozolol and ascorbic acid in hydrogel on midline laparatomy wound healing in a diabetic mouse model Oral administration of marine collagen peptides prepared from chum salmon (*Oncorhynchus keta*) improves wound healing following cesarean section in rats Whey peptides improve wound healing following caesarean section in rats	5
Wound healing not evaluated	Acceleration of soft tissue repair by a thrombin-derived oligopeptide Annexin A12-26 treatment improves skin heterologous transplantation by modulating inflammation and angiogenesis processes Blood vessel occlusion in peri-burn tissue is secondary to erythrocyte aggregation and mitigated by a fibronectin-derived peptide that limits burn injury progression Effect of peptide bioregulator on healing of excision wounds in old animals	4
Total		**108**

S3 Table. General characteristics of the preclinical models used in all studies investigating the relevance of animal peptides in the treatment of skin wounds.

Reference	Animal models					
	Animal	**Strain**	**Sex**	**Age**	**Weight**	**Associated pathology**
[20]	Rat	?	?	?	?	No
[21]	Rat	Sprague-Dawley	♂	?	250-300 g	No
[22]	Rat	Sprague-Dawley	♂	?	280-340 g	Ischemia
[23]	Mice	C57BL/6	?	?	?	No
[15]	Rat	Sprague-Dawley	♂	?	230-250 g	No
[24]	Mice	C57BL/6	?	8-12 wk	?	No
[25]	Mice	Balb/C	♂	?	?	No
[26]	Mice	Balb/C	♀	6-8 wk	?	No
[27]	Mice	Kunming	♂	6-8 wk	?	No
[28]	Mice	Balb/C - *db*/*db*	♂	8 wk	?	Diabetes
[29]	Rat	Wistar	♂	43 wk	500-600 g	No
[30]	Mice	Balb/C	♀	6-8 wk	?	No
[12]	Mice	Balb/C	♀	6-8 wk	?	No
[31]	Mice	C57BL/6	♂	6 wk	?	Diabetes
[32]	Rat	Sprague-Dawley	♂	8 wk	150-200 g	Diabetes
[33]	Mice	Kunming	♂	7-8 wk	?	No
[34]	Pig	Yorkshire	?	6 wk	10-13 kg	No
[35]	Rabbit	?	♂♀	?	?	No
[1]	Mice	?	♂	?	22-25 g	No
[13]	Mice	?	♂	?	22-25 g	No
[16]	Mice	?	♂	?	22-25 g	No
[36]	Mice	Kunming	♂	6-7 wk	?	No
[37]	Rat	Sprague-Dawley	♂	?	160-170 g	No
[38]	Mice	?	♂	?	20-25 g	No
[39]	Mice	?	♂	?	26 g	No
[40]	Mice	?	♂	?	22-25 g	No
[41]	Mice	Balb/C	♀	?	18-20 g	No
[42]	Mice	?	?	?	?	No

[43]	Rat	Sprague-Dawley	♂	?	150-170 g	No
[44]	Mice	Kunming	♀	6 wk	27-30 g	No

♂: Male, ♀: Female, ?: Not reported or unclear, wk: Weeks.

S4 Table. General characteristics of skin wounds used in preclinical models investigating the relevance of animal peptides as healing agents.

Reference	Cutaneous wounds				
	Lesion	**Site**	**Initial area**	**Number**	**Infection/ Concentration**
[20]	Excision	Dorsum	8 mm D	6	Uninfected
[21]	Excision	Dorsum	2 cm D	2	Uninfected
[22]	Excision	Dorsum	2 cm D	2-4	Uninfected
[23]	Excision	Dorsum	3 mm D	1	Uninfected
[15]	Excision	Dorsum	2 cm D	2	Uninfected
[24]	Excision	Dorsum	5 mm D	2	Uninfected
[25]	Excision	Dorsum	7 mm D	?	Uninfected
[26]	Excision	Abdomen	1 cm D	1	*S. aureus*/10^6 CFU
[27]	Excision	Dorsum	9 mm D	2	Uninfected
[28]	Excision	Dorsum	7 mm D	?	Uninfected
[29]	Excision	Dorsum	2 cm^2	1	Uninfected
[30]	Excision	Dorsum	1 cm D	1	*S. aureus*/10^6 CFU
[12]	Excision	Dorsum	1 cm D	1	*S. aureus*/10^6 CFU
[31]	Excision	Dorsum	4 mm D	?	Uninfected
[32]	Incision	Dorsum	2×5 mm	1	Uninfected
[33]	Excision	Dorsum	1.44 cm^2	1	Uninfected
[34]	Burn	Dorsum	3 cm D	6	*S. aureus*/10^{10} CFU
[35]	Burn	Dorsum	4 cm^2	2	Uninfected
[1]	Excision	Dorsum	8×8 mm	2	Uninfected
[13]	Excision	Dorsum	8×8 mm	2	Uninfected
[16]	Excision	Dorsum	8×8 mm	2	Uninfected
[36]	Excision	Dorsum	6 mm D	2	Uninfected
[37]	Excision	Dorsum	1 cm D	1	Uninfected
[38]	Excision	Dorsum	8×8 mm	2	Uninfected
[39]	Excision	Dorsum	8.5 mm D	1	Uninfected
[40]	Excision	Dorsum	8×8 mm	2	Uninfected
[41]	Excision	Dorsum	8 mm D	1	Uninfected

[42]	Excision	Dorsum	0.8 cm D	1	Uninfected
[43]	Excision	Dorsum	1 cm	1	Uninfected
[44]	Excision	Dorsum	1 cm^2	1	*E. coli*/2 x 10^5 CFU

?: Not reported or unclear, *S. aureus*: *Staphylococcus aureus*, *E. coli: Escherichia coli*, D: Diameter, CFU: Colony forming unit.

S5 Table. Description of the main characteristics related to peptides included in the systematic review on peptides of animal origin applied in the treatment of skin wounds.

	Peptides		
Reference	**Name**	**Origin**	**Sequence**
[20]	Thymosin β4	Bovine thymus	?
[21]	TP508	Human thrombin	AGYKPDEGKRGDACEGDSGGPFV
[22]	TP508	Human thrombin	AGYKPDEGKRGDACEGDSGGPFV
[23]	HB-107	*Hyalophora cecropia*	MPKEKVFLKIEKMGRNIRN
[15]	Marine collagen peptides (MCP)	*Oncorhynchus keta*	?
[24]	LL37	Human cathelicidin	LLGDFFRKSKEKIGKEFKRIVQRIKD FLRNLVPRTES
[25]	AH90	*Odorrana grahami*	ATAWDFGPHGLLPIRPIRIRPLCG
[26]	Pardaxin (GE33)	*Pardachirus marmoratus*	?
[27]	Tylotoin	*Tylototriton verrucosus*	KCVRQNNKRVCK
[28]	CW49	*Odorrana grahami*	APFRMGICTTN
[29]	E1	Bovine achilles tendon	GETGPAGPAGPIGPVGARGPAGPQ GPRGDKGETGEQ
[30]	Tilapia piscidin 4 (TP4)	*Oreochromis niloticus*	FIHHIIGGLFSAGKAIHRLIRRRRR
[12]	Tilapia piscidin 3 (TP3)	*Oreochromis niloticus*	FIHHIIGGLFSVGKHIHSLIHGH
[31]	Proinsulin C	Human proinsulin	?
[32]	Camel milk peptide (CMP)	Camel milk	?
[33]	Ghrelin	Mouse ghrelin	?
[34]	Epinecidin-1 (Epi-1)	*Epinephelus coioides*	GFIFHIIKGLFHAGKMIHGLV
[35]	Marine collagen peptides (MCP)	*Oreochromis niloticus*	?
[1]	OM-LV20	*Odorrana margaretae*	LVGKLLKGAVGDVCGLLPIC
[13]	Cathelicidin-OA1	*Odorrana andersonii*	IGRDPTWSHLAASCLKCIFDDLPKTH N
[16]	OA-GL21	*Odorrana andersonii*	GLLSGHYGRVVSTQSGHYGRG
[36]	Cathelicidin-NV	*Nanorana ventripunctata*	ARGKKECKDDRCRLLMKRGSFSYV
[37]	Pollock Collagen Peptide (PCP)	*Theragra chalcogramma*	?
[38]	OA-FF10	*Odorrana andersonii*	FFTTSCRSC
[39]	Collagen peptides (CP1/CP2)	*Rhopilema esculentum*	?
[40]	OA-GL12	*Odorrana andersonii*	GLLSGINAEWPC
[41]	Ot-WHP	*Odorrana tormota*	ATAWDLGPHGIRPLRPIRIRPLCG
[42]	Active peptides (APs)	*Pinctada martensii*	?
[43]	Skin collagen peptide (SCP)	*Salmo salar* and *Tilapia nilotica*	?
[44]	Cathelicidin-DM	*Duttaphrynus melanostictus*	SSRRKPCKGWLCKLKLRGGYTLIG SATNLNRPTYVRA

S6 Table. Treatment protocols used in all studies investigating the relevance of animal peptides in the treatment of skin wounds.

	Intervention				
Reference	**Vehicle**	**Route**	**Application**	**Treatment time (days)**	**Concentration**
[20]	SAL	Topic I.p.	Twice Every two days	7	5 µg/50µl 60 µg/300µl
[21]	SAL	Topic	Single	?	0.03, 0.1, 0.3, 0.4, 3 and 5 µg
[22]	SAL	Topic	Single	?	0.1 µg
[23]	PBS	Topic	Three times per day	14	100 µg/ml
[15]	Water	Oral	Once daily	?	2 g/kg
[24]	Water	Topic	Twice daily	7	10 µg
[25]	DPBS	Topic	Twice daily	?	250 µg/ml
[26]	PBS	Topic	?	?	8 mg/ml
[27]	PBS	Topic	Twice daily	?	20 µg/ml
[28]	DPBS	Topic	Twice daily	?	200 µg/ml
[29]	SAL	Topic	Once daily	12	60 µM
[30]	PBS	Topic	?	?	2 mg/ml
[12]	PBS	Topic	?	?	2 mg/ml
[31]	PBS	S.c.	Continuous	14	35 pmol/kg/min
[32]	?	Oral	Once daily	7	25 mg/kg
[33]	PBS	S.c.	Once daily	7	50, 100 and 200 nmol/kg
[34]	PBS	Topic	Every three days	25	90, 900 µg/ml and 9 mg/ml
[35]	?	Topic	Once daily	28	?
[1]	SAL	Topic	Twice daily	?	0.5, 1, 2.5, 5, 10 and 20 nM
[13]	SAL	Topic	Twice daily	?	10, 20 and 40 µM
[16]	SAL	Topic	Twice daily	?	1, 10 and 100 µg/ml
[36]	?	Topic	Twice daily	?	200 µg/ml
[37]	SAL	Oral	?	7	0.5 and 2 g/kg
[38]	SAL	Topic	Twice daily	?	1, 10 and 100 µM
[39]	SAL	Oral	Once daily	6	0.3, 0.6 and 0.9 g/kg
[40]	SAL	Topic	Twice daily	?	0.1, 1 and 10 nM
[41]	PBS	Topic	Once daily	8	200 µg/ml
[42]	SAL	Oral	?	?	0.5 and 2 g/kg
[43]	SAL	Oral	?	?	2 g/kg
[44]	PBS	I.v.	Once daily	5	10 mg/kg

?: Not reported or unclear, SAL: Saline solution, PBS: Phosphate buffered saline solution, DPBS: Dulbecco's phosphate-buffered saline, I.p.: Intraperitoneal, S.c.: Subcutaneous, I.v.: Intravenously.

S7 Table. PRISMA 2009 Checklist. *From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	-
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4-5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4-5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4-5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4-5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	4-5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	4-5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	-
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	-

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	-
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	5-6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	5-16
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	17
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	5-16
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	-
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	-
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	-
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	18-20
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	21
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	21
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	21

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097.

For more information, visit: **www.prisma-statement.org**.

3. ARTIGO II

SKIN PEPTIDE FRACTION FROM *Lithobates catesbeianus* (SHAW, 1802) SUPPRESSES INFLAMMATION AND OXIDATIVE STRESS IN THE WOUND HEALING PROCESS IN MICE

Abstract

Skin lesions represent a serious health public problem, reducing the patient's quality of life and causing high costs for health systems around the world. Bioactive peptides of animal origin are being explored as potential therapeutic agents for the treatment of skin wounds, due to their pharmacological activity, low cost, easy availability, and storage. The present study aimed to analyze the effect of a peptide fraction obtained from bullfrog skin by enzymatic hydrolysis in the healing of second intention cutaneous wounds in mice. To extract peptides from bullfrog skin, enzymatic hydrolysis was performed using four different enzymes separately (alcalase, pepsin, papain and trypsin). The proliferative capacity of the hydrolysates was evaluated in RAW 264.7 macrophage cell culture. Among the hydrolysates, trypsin derived hydrolysate exhibited the highest proliferative activity than the other enzymatic hydrolysates and a dose-dependent effect. The trypsin derived hydrolysate was then fractionated by solid-phase extraction and the fraction with the highest proliferative effect (fraction F4) was tested in RAW 264.7 macrophage and NIH/3T3 fibroblasts cells at different concentrations (3.125, 6.25, 12.5, and 25 µg/mL) and used in the wound healing experiment in mice. Mass spectrometry analysis of the fraction F4 was performed to investigate the composition of the peptides present in this fraction. Forty-two male mice of the BALB/C strain were randomly divided into three groups of 14 animals each (protocol no. 824/2018), and a skin wound (12 mm diameter) was created on the back of each animal. Two *in vitro* tested doses of the fraction F4 (3.125 and 25 µg/mL) were selected for the *in vivo* experiment. The groups were as follows: C, received distilled water (control); L1, peptides at a concentration of 3.125 µg/mL; and L2, peptides at a concentration of 25 µg/mL. Topical applications were performed daily for a period of 12 days, and dermal biopsies were collected on the day of injury and on days 6 and 12 post-injury. Our *in vitro* analysis demonstrated that the peptides from fraction F4 led to RAW 264.7 macrophages and NIH/3T3 fibroblasts proliferation. In the mass spectrometry analysis, only low molecular weight peptides were identified in the fraction F4. In addition, 71 peptide sequences related to bullfrog proteins were identified. Our *in vivo* findings showed that the proliferative effect observed in the *in vitro* study also occurred in scar tissue, with there being an increase in the number of cells labeled with the Ki67 antibody. In addition, there was a reduction in the number of total macrophages and M2 macrophages. The toll-like receptor 4 (TRL-4) pathway was inhibited, and consequently, there was a reduction in the interleukin-6 (IL-6) and interleukin-1β (IL-1β). The cyclooxygenase-2 (COX-2) pathway expression also decreased after exposure to peptides of the fraction F4, promoting a reduction in the inflammatory phase time, and favoring the healing process. The peptides of the fraction F4 showed a high antioxidant power because there was an increase catalase activity and the malondialdehyde and carbonyl proteins levels were reduced in the tissue. Finally, peptides of the fraction F4 promoted the reorganization in the extracellular matrix by increasing types I collagen fibers number accelerating the skin wounds closure. Our findings indicated that the treatment with peptides of the fraction F4 potentiates the healing process, with the best effects being found in the administration of the highest concentration.

Keywords: Wound healing, Peptides, Cell proliferation.

Introduction

The healing process is a complex biological process of repairing injured tissue, characterized by the activation of different cells, and mechanisms to restore the tissue integrity after injury [1]. This process can be divided into three phases, known as inflammatory phase, cell proliferation, and tissue remodeling, which involves a complex interaction between cells, extracellular matrix components, cytokines, and growth factors [2]. At the beginning of the healing process, the collagen exposed during injury initiates the coagulation cascade, which results in the formation of a clot composed of collagen, platelets, thrombin, fibronectin, and cytokines, contributing to the inflammatory response [3]. Chemotactic cytokines produced during this stage induce the migration of immune system cells to the site of injury [4]. Neutrophils are the predominant cells in the initial phases of inflammation, later monocytes are attracted and mature into macrophages, which play important roles in all phases of the healing process [5]. Macrophages can be phenotypically divided into M1 and M2. The M1 proinflammatory macrophage cells release interferon-γ (IFN-γ), IL-1β, IL-6, and COX-2 that promote the inflammatory process, and the M2 anti-inflammatory macrophage cells act mainly in the later stages to solve the inflammatory phase [6]. All these cells migration is orchestrated and regulated by different pathways, in which we can highlight Toll-like receptors (TLRs), particularly TLR-4 and its downstream signaling myeloid differentiation primary response protein 88 (MyD88) pathway [7]. This pathway plays an important role in the inflammatory phase of the wound healing process [8]. Since an initial inflammatory phase is very important for tissue repair a precise timing of both the initiation and resolution of inflammation is essential for restoring tissue integrity.

At the next phase, immune cells at the site of injury provide a favorable microenvironment for cell migration and proliferation [5]. The fibroblasts that migrate and proliferate at the site of injury will synthesize important components of the extracellular matrix and give rise to granulation tissue [9]. Transforming growth factor-β (TGF-β), highly produced and released during the inflammatory phase, is one of the main molecules involved in the induction of fibroblast differentiation into myofibroblasts, responsible for the synthesis of type I collagen, type III collagen, fibronectin and wound contraction [10]. The initially formed granulation tissue is composed of an infiltrate of cells, blood vessels, and mainly type III collagen fibers, which helps to protect the wound from further damage [11]. The final phase of the healing process is characterized by a decrease in the number of cells, blood vessels and mainly by changes in the extracellular matrix of the scar tissue, with the replacement of type III collagen by type I collagen [12]. This process is carried out through a tightly controlled synthesis of new collagen and lysis of old collagen, performed mainly by the action of matrix metalloproteinases (MMPs) [13].

The normal healing process can be affected by several external and internal factors, resulting in delay and impairment of the tissue recovery process, increasing costs for the treatment of injuries [14]. During the inflammatory phase, large amounts of reactive oxygen species (ROS) are produced by NADPH oxidase, an enzymatic complex, which is expressed at high levels by inflammatory cells [15]. ROS are important defenses against pathogens, however, if ROS detoxification is insufficient or if ROS are produced in excessive amounts, oxidative stress occurs, resulting in severe cellular damage and delay in the healing process [16]. Enzymes of the antioxidant defense system such as superoxide dismutase, catalase, and glutathione are produced to reduce the damage caused by oxidative stress and promote the wound healing process [17].

In the search for a treatment that acts on the different pathways of the cicatricial process mentioned, several types of treatments for skin injuries have been researched, but generally are not effective to promote fast wound closure [18]. In addition, the formulations therapeutic

available today has a high cost, adverse effects and bacterial resistance [19]. Therefore, many compounds of animal origin are being studied for the acceleration of wound closure. Amphibian skin has a wide source of bioactive peptides, which based on their structural, compositional, and sequential properties may have relevant effects on the healing process [19–21]. The bullfrog *Lithobates catesbeianus* (Shaw, 1802) is a species native to North America, from southern Quebec in Canada to the eastern United States, it has a relatively large body size with rapid sexual development and the ability to reach high population density [22]. It was introduced for commercial cultivation in Brazil and several parts of the world due to the great appreciation of its meat in cooking [23]. Bullfrog skin has always been considered a by-product of little use and is generally regarded as waste [24]. However, the skin of this animal has already been used as a temporary occlusive dressing for the treatment of severe burns, contributing to better healing results [25]. In this context, this study aimed to evaluate the effect of peptide fractions obtained from bullfrog skin by enzymatic hydrolysis on the cell proliferation *in vitro* and understand what is the influence of these peptides on the TLR-4 receptor signaling during the inflammatory phase of the wound healing process in a preclinical model.

Materials and methods

Sample collection and animal care

In this study, ten specimens of the bullfrog (males, 250 ± 15g, and 38 weeks old) from the Experimental Frog Farm of the Federal University of Viçosa (UFV) were used. Environmental conditions were controlled for animals to adapt for 3 days (temperature: 27 ± 1 °C and light/dark cycle: 12/12 h). The frogs were fed twice per day, using commercial extruded feed with 44g of crude protein and 1% of *Musca domestica* (Linnaeus, 1758) larvae as an attractant. The animals were weighed and euthanized with sodium thiopental (Thiopentax) subcutaneously. After euthanasia, the skins were separated and washed in sterile saline solution (0.9% NaCl solution, previously cooled to 4 °C), and then stored in a freezer at -20 °C until further use. All the experiments involving animals were approved by the Animal Ethics Committee of the Federal University of Viçosa (registration no. 824/2018). In order to describe the general process of our experiment, it is presented in S1 Figure the flowchart of the steps performed throughout the experiment.

Preparation of bullfrog skin hydrolysates

To extract peptides from bullfrog skin, enzymatic hydrolysis was performed using four different enzymes separately (alcalase, pepsin, papain, and trypsin), respecting their optimal conditions of activity [26]. At an enzyme/substrate ratio of 1/100 (w/w), 1% substrate and enzyme were mixed. The mixture was incubated for 8 h at each optimal temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. The hydrolysates were lyophilized and tested for proliferative activity in RAW 264.7 macrophage cell culture at different concentrations (25, 50, 100, and 200 μg/mL). The hydrolysate with the highest proliferative effect was selected for fractionation.

Solid-phase extraction

The peptides fractionation process was carried out by solid-phase extraction, where 10 mg of the lyophilized extract were solubilized in 1 mL of distilled water and then centrifuged at 10,500 × *g* for 10 min. The supernatant was collected and injected into a Whatman® ODS-5 solid-phase extraction column. Elution was performed using a gradient of 0-85% acetonitrile in

0.1% trifluoroacetic acid, so 10 fractions were obtained (F1 = 0%, F2 = 5%, F3 = 15%, F4 = 25%, F5 = 35%, F6 = 45%, F7 = 55%, F8 = 65%, F9 = 75%, and F10 = 85%). A bioactivity indicator assay was performed using a volume of 2 mL of each fraction, which were lyophilized and tested for proliferative activity in RAW 264.7 macrophage cell culture. The fraction with the highest proliferative effect was tested in RAW 264.7 macrophage and NIH/3T3 fibroblasts cells at different concentrations (3.125, 6.25, 12.5, and 25 µg/mL) and used in the *in vivo* wound healing experiment in mice. Part of the fraction was dried in a vacuum centrifuge and stored in a -20°C freezer until it was analyzed by mass spectrometry.

Cell proliferation assay

Cell proliferation tests performed on RAW 264.7 macrophage and NIH/3T3 fibroblasts were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method [27]. Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin/streptomycin in a humidified 5% CO_2 37 °C incubator. To evaluate the effect of the peptides on cell proliferation, RAW264.7 macrophages and NIH/3T3 fibroblasts were seeded into 96-well plates at a density of $2x10^4$ cells/well in 200 µL medium. After 24 h, the supernatant was replaced with a new culture medium containing the peptides at different concentrations, and the incubation continued for the next 22 h. The control (100% of growth) was carried out with cells cultured in medium only. After the incubation period, 50 µL of the supernatant was removed and 50 µL of MTT solution (0.5 mg/mL) was added to each well, and the cells were incubated again for a period of 2 h. After this period, 90 µL of supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to the wells. The absorbance was measured on microplate reader (Multiskan FC, Thermo Labsystems, Franklin, MA, USA) set to 570 nm. The experiments were performed in triplicate.

Mass spectrometry

The fraction of interest was solubilized in 20 µL of 0.1% aqueous formic acid solution, added 2% (v/v) of LCMS grade acetonitrile, packed in appropriate tubes for application in the nano LC-MS/MS system used. After sample preparation, 1 µL of the solution was injected and analyzed by nano LC-MS, using the ultra-high performance liquid chromatograph – UHPLC NanoAcquity (Waters, USA), containing a trap column model nanoAcquity UPLC® 2G-V/MTrap 5 µm Symmetry® C18 180 µm x 20 mm, at a flow rate of 7 µL/min, for 3 minutes. Peptides were separated by a nanoAcquity UPLC® 1.8µm HSS T3 75 µm x 200 mm column, operating at a flow rate of 0.2 µL/min. The mobile phase of the chromatographic process had water acidified with 0.1% formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B) as solvents. The chromatographic separation took place according to the following schedule: 2% B for 1 min; 2% to 30% B gradient for 209 min; 30% to 85% B gradient for 10 min; maintenance at 85% B for 5 min; 85% to 2% B for 5 min; and maintenance at 2% B for 10 min, totaling 240 minutes of chromatographic analysis. The eluted peptides were automatically injected into a MAXIS 3G mass spectrometer (Bruker Daltonics, Germany), operating in online mode, with a CaptiveSpray ionization source. The peptides were analyzed using an appropriate method for proteomic analysis (IE_captive_nov2019), with a drying gas flow of 3 L/min, ionization source temperature of 150 ºC and transmission voltage of 2 kV. The raw data were converted into a list of masses in the extension *mzXML (Extensible Mark-up Language), using the CompassXport software version 3.0 (Bruker Daltonics, Germany), which was subjected to identification using the PEAKS software.

Analysis of data from mass spectrometry

The lists of masses with extension *mzXML were compared with the database of protein sequences of the specie *Lithobates catesbeianus* (downloaded on 01/31/2022, with 29,178 entries), deposited at the Uniprot Consortium. The comparison was performed using the PEAKS software version 7.0 (Bioinformatics Solutions Inc., Canada) [28]. The parameters used in this study were: enzymatic digestion by trypsin, considering the occurrence of two missed cleavages; cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. Error tolerance for the parental ion of 20 ppm and 0.6 Da for the fragments, considering the analysis of ions with charges +2, +3, and +4. Proteins were considered identified when they presented at least two unique peptides with FDR (False Discovery Rate) less than one percent. Proteins identified as "uncharacterized" were analyzed using BLAST software version 2.4.0 [29]. In this analysis, it was possible to identify which proteins deposited in the non-redundant (nr) protein database from National Center of Biotechnology Information (NCBI) showed greater identity with the sequences of the "uncharacterized" proteins.

***In vivo* Assays**

Animals

Forty-two BALB/c mice (*Mus musculus*) (males, 35 ± 1g, and 12 weeks old), were obtained from the Central House of the Health and Biological Sciences Center, UFV. These animals were randomly allocated in individual cages, which were cleaned daily and maintained under controlled environmental conditions (temperature: 22 ± 1 °C and light/dark cycle: 12/12 h). Commercial Food and water were provided *ad libitum*. The animals were kept under these conditions for an adaptation period of 7 days before the beginning of the experiments.

The procedure of surgical wounds

The mice were anesthetized with an intraperitoneal injection of sodium pentobarbital. After anesthesia, the dorsolateral shaving of the animals was performed, and the area was cleaned with 70% alcohol. The dorsum region was demarcated, corresponding to an area of 12 mm in diameter, a single wound was made in the dorsal region of each mouse using surgical scissors. Tissue biopsies were collected for histological analysis (day 0). The animals were randomized into three groups with 14 animals in each group: C (distilled water, control), L1 (peptides extracted from bullfrog skin at a concentration of 3.125 µg/mL in distilled water), and L2 (peptides extracted from bullfrog skin at a concentration of 25 µg/mL in distilled water). Topical administration of treatments was performed once a day for 12 days in a volume of 25 µL. After 6 days of treatment, half of the animals in each group were anesthetized and scar tissue biopsies were collected in the same way as described above, later the animals were euthanized by cardiac puncture and exsanguination. These processes were carried out in the other animals at the end of 12 days.

Calculation of the area and the rate of wound contraction

The area and rate of contraction of the wound were evaluated every 6 days, using digital images obtained by an LG-M400 smartphone (LG Electronics, Busan, KOR). To measure the wound area, the images were analyzed using ImageJ software version 1.53a (National Institutes of Health, Rockville, MD, USA). Support to put the camera was used to guarantee the exact

distance between the camera in the animals' dorsum. The wound contraction rate was calculated by the ratio: initial wound area (A_o) – the area on a given day (A_i)/initial wound area (A_o) x 100 [30].

Histological and stereological analysis

Skin fragments for histological analysis were fixed in a 10% neutral buffered formalin solution for a period of 12 h, dehydrated in ethanol, diaphanized in xylene, and immersed in paraffin. Histological sections (3 μm thick) were obtained on a Leica Multicut 2045 rotary microtome (Reichert-Jung Products, Germany). We used 1 of every 15 sections to avoid repeating the analysis of the same histological area. The sections were stained with hematoxylin and eosin (HE) for the analysis of the cellularity and blood vessels [31]. Sirius red staining was used to differentiate type I and III collagen fibers by polarization microscopy [32]. Toluidine blue was used to identify and count mast cells [33]. The slides were visualized and captured in Leica DM 750 microscope coupled with a Leica MC 170 HD digital camera (Leica Microsystems, Wetzlar, Germany). Five images were selected at random using a 20x objective lens. Histomorphometry was performed using Image-Pro Plus software version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA). For this analysis, a grid containing 304 points was superimposed over each image. The stereological parameters of volumetric density (Vv) were calculated by counting the points that occurred over cells, blood vessels, and types I and III collagen fibers, using the ratio: Vv = PP/PT, where PP is the number of points occurring over the structures of interest and PT is the total number of points on the test system [31,34]. Collagen fibers were analyzed according to the different properties of birefringence, as thick collagen fibers (type I) appear in shades of colors ranging from red to yellow, whereas type III collagen fibers show a bright green color under polarization [31,32]. The mast cells were analyzed using a 40x objective lens. Ten microscopical fields were randomly analyzed in each histological section to obtain a total area (TA) of 1,96 mm^2. The number of mast cells per unit of histological area was calculated using the ratio: QA = ∑mast cells/TA [35].

Immunostaining

The fragments for immunofluorescence analysis were fixed in 4% paraformaldehyde solution for 60 min. After fixation, the fragments were washed three times for 1 h each in saline sodium phosphate buffer (PBS 0.1 M, pH 7.2) in 1% Tween 20, followed by incubation for 24 h at 4°C in the following primary antibody solutions: anti-COX-2 for cyclooxygenase-2 (1:1000 dilution, Abcam: ab15191), anti-F4/80 for total macrophages (1:100 dilution, Abcam: ab111101), anti-CD163 for M2 macrophages (1:500 dilution, Abcam: ab1824222), and anti-Ki67 for cell proliferation (ready solution, Abcam: ab21700). After incubation, the samples were washed three times (10 min each) with PBS and incubated for 24 h at 4 °C with FITC-conjugated secondary antibodies (Sigma-Aldrich Corp., St Louis, MO, USA.) (1:500 dilution), followed by three washes with PBS 10 min each. Samples were dehydrated in ethanol and embedded in Leica historesin. The sections (3 μm thick) were obtained on a Leica RM 2255 rotary microtome (Leica, Wetzlar, Germany), using glass knives. The nuclei were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (1:1000 dilution). The slides were visualized and captured in EVOS M5000 microscope (Thermo Fisher Scientific, Pudong, Shanghai, China), using a 20x objective lens. Cell counts were performed using the ImageJ software version 1.53a (National Institutes of Health, Rockville, MD, USA).

Biochemical assays

All tissue fragments for analysis of oxidative stress and antioxidant enzyme activity were immediately frozen in liquid nitrogen (-196 °C) and kept in a -80 °C ultra-freezer until further analysis. The samples (150 mg) were homogenized in 1.5 mL of 0.2 mol/L phosphate buffer, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4 using a homogenizer [36]. The homogenates were centrifuged at 15,000 × *g* for 10 min at 4 °C and the supernatants were used for the analyzes of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione S-transferase (GST), and catalase (CAT). The resulting pellets were used for the analysis of carbonyl proteins (CP). Biochemical data were normalized according to the total protein levels in the supernatant.

Lipid and protein oxidation

Lipid peroxidation was determined by measuring levels of total malondialdehyde, which is one of the main products of lipid peroxidation and is commonly used as a marker of oxidative stress [37]. The MDA concentration was determined by using the standard curve of known concentrations of 1, 1, 3, 3-tetramethoxypropane (TMPO). The results were expressed as μmol/L/mg protein. Protein oxidation was estimated from protein carbonyl content, which was measured using 2,4-dinitrophenylhydrazine (DNPH) [38], based on the carbonyl group reaction with DNPH. The results were expressed as nmol/mL.

Antioxidant enzyme activity and total protein

The activity of SOD was determined by the superoxide and hydrogen peroxide reduction method, thereby decreasing the autooxidation of pyrogallol [39]. SOD activity was calculated as units (U)/mg protein, with one unit of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. GST activity was analyzed according to the formation of 2,4-dinitrochlorobenzene (CDNB) conjugated to glutathione [40]. One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of one μmol of product/min/mL. The CAT activity was performed using hydrogen peroxide as a substrate [41]. The degradation of hydrogen peroxide was evaluated for 3 min and then ammonium molybdate was added to stop the reaction. The results were expressed as U/mg protein. Total protein assay was performed by a method involving binding of the Coomassie Brilliant Blue Protein G-250 dye to the protein [42], using bovine serum albumin (BSA) as a standard.

Total RNA extraction, cDNA synthesis, and PCR analysis

Total RNA from dermal biopsies was extracted using the standard TRIzol™ protocol and cDNA was synthesized using oligo dT (15) and random primers with GoScript™ Reverse Transcription system (Promega Corporation, Madison, WI), according to the manufacturers' instructions. To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA) as follows: TLR-4, forward primer 5′-GGA CTC TGA TCA TGG CAC TG-3′, reverse primer 3′-CTG ATC CAT GCA TTG GTA GGT-5′; IL-1β, forward primer 5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′, reverse primer 5′-GAT CCA CAC TCT CCA GCT GCA-3′; IL-6, forward primer 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer 5′-TTG GTC CTT AGC CAC TCC TTC-3′; and COX-2, forward primer 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse primer 5′-GTG GTA ACC GCT CAG GTG TTG-3′. Quantitative PCR (qPCR) amplifications were performed on an ABI 7500

Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. The mRNA expressions of the control and peptide-treated groups were compared.

Statistical analysis

The statistical analysis was carried out using the GraphPad Prism 7 software (GraphPad Software Inc., San Diego, Calif., USA). The results were expressed as mean and standard deviation (mean ± SD). The data was first evaluated for normality using a Shapiro Wilk test, and after it was confirmed to be normally distributed, data were compared using a one-way ANOVA with Tukey's t-test for post hoc analysis. When the distribution was not considered normal, the data were submitted to the Kruskal-Wallis test followed by the Dunn's test. Statistical significance was set at 5% ($p < 0.05$).

Results

Effect of peptides on cell proliferation *in vitro*

The hydrolysates obtained from bullfrog skin using four different enzymes separately demonstrated positive effects on cell proliferation of RAW 264.7 macrophages at different concentrations (Figure 1a). However, the trypsin derived hydrolysate showed the greatest effect on cell proliferation among the four hydrolysates tested (122.7 ± 4.04% cell viability at 200 µg/mL) and dose-dependent response, being selected for fractionation by solid-phase extraction. Fractionation by solid-phase extraction proved to be an efficient technique to separate the compounds present in the trypsin derived hydrolysate, with three fractions showing cytotoxic effects (F2, F9, and F10), five fractions no effects on cell viability (F1, F3, F5, F7, and F8), and two fractions (F4 and F6) increasing cell proliferation in RAW 264.7 macrophages (Figure 1b). The results reveal that the fraction F4 demonstrated the greatest proliferative effect (140 ± 3.51% cell viability). Therefore, we catch the fraction F4 and evaluated it different concentrations and observed an increase in the cell proliferation in RAW 164.7 macrophages (178 ± 13.45% cell viability at 25 µg/mL) (Figure 1c) and NIH/3T3 fibroblasts too (198 ± 11.53% cell viability at 25 µg/mL) (Figure 1d). Our results showed an effect dose-response with the best results at 25 µg/mL. The fraction F4 was then selected for *in vivo* experiments and mass spectrometry analysis.

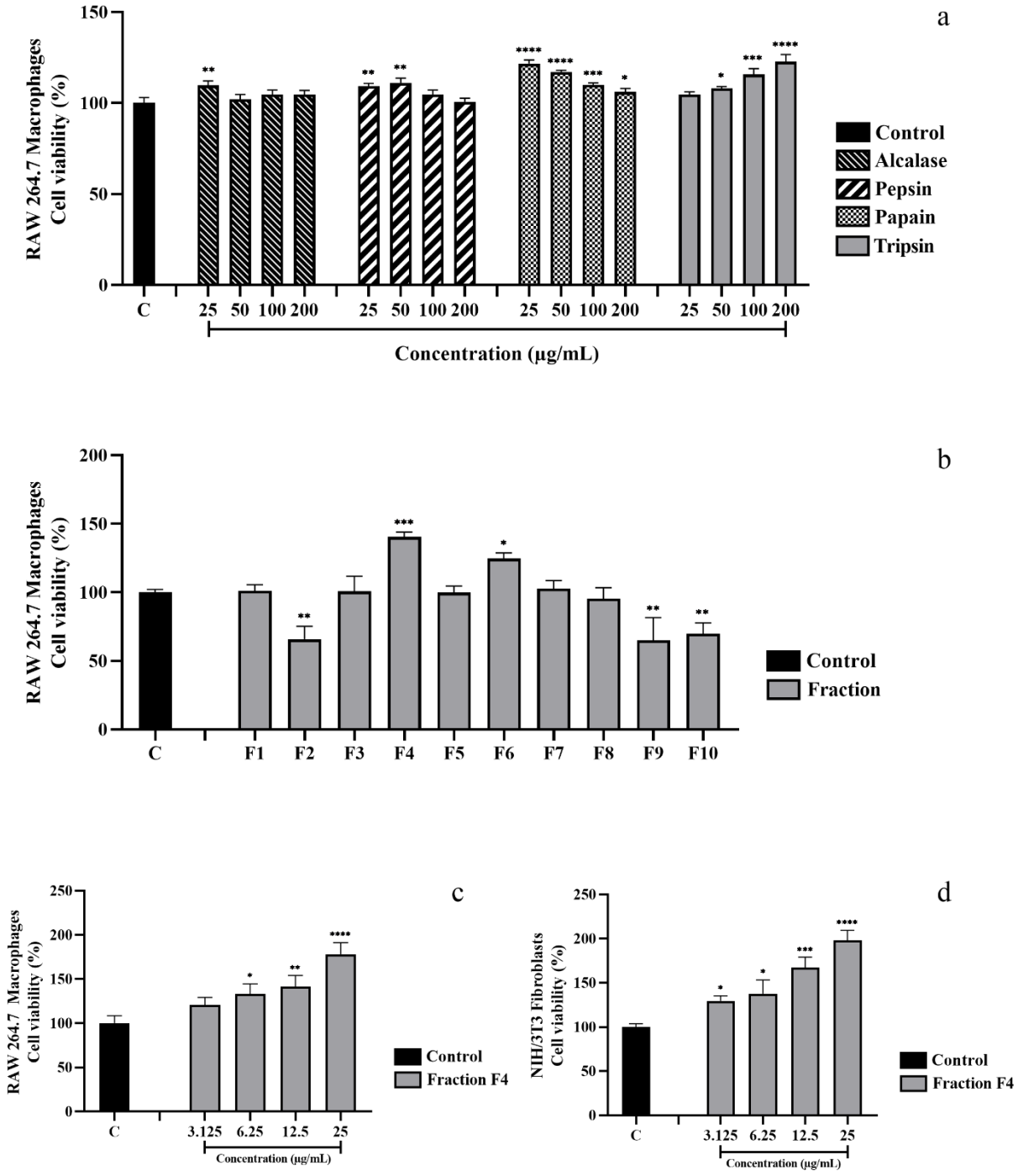

Figure 1. Effect of peptide fractions on cell viability. RAW264.7 macrophages were treated separately with hydrolysates from bullfrog skin using four different enzymes separately (alcalase, pepsin, papain and trypsin) at different concentrations (25, 50, 100, and 200 µg/mL) for 24 h (a). RAW 264.7 macrophages were treated separately with ten fractions obtained from the trypsin derived hydrolysate, which 2 mL of each fraction was lyophilized, resuspended in culture medium and applied to the cells for 24 h (b). RAW 264.7 macrophages were treated with the fraction F4 at different concentrations (3.125, 6.25, 12.5, and 25 µg/mL) for 24 h (c). NIH/3T3 fibroblasts were treated with the fraction F4 at different concentrations (3.125, 6.25, 12.5, and 25 µg/mL) for 24 h (d). The data (n = 3) are expressed as mean and standard deviation. Statistical difference *vs.* control group *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

Mass spectrometry results

Mass spectrometry analysis showed only low molecular weight peptides (< 5 kDa) in the fraction F4. In addition, 71 peptide sequences were related to bullfrog proteins such as: collagen alpha-1 (I) chain, collagen alpha-1 (XII) chain isoform X3, collagen alpha-2 (I) chain, collagen alpha-3 (VI) chain isoform X6, collagen alpha-3 (VI) chain isoform X11, hemoglobin subunit alpha, hemoglobin subunit beta, monomeric alpha-macroglobulin, albumin, vimentin-4, annexin, tropomyosin-1 alpha, fibrinogen C-terminal, actin cytoplasmatic 2, AhpC-TSA, 2-phospho-D-glycerate hydro-lyase, histone H4, and intermediate filament rod domain. Details of the peptide sequences, their respective masses and related proteins are available in S1-S2 Tables. Peptide sequences that are not completely aligned with the proteins can be provided upon request.

Wound area and the rate of wound contraction

The wound area was significantly smaller in the groups treated with peptides of the fraction F4 compared to the control group at day 12 post-injury, with the L2 group showing greater wound area reduction effects in comparison to the L1 group (Figure 2a). The highest rates of wound contraction were observed on day 12 in the L1 and L2 groups (71.56 ± 4.45% and 87.52 ± 2.75%, respectively) compared to the control group (58.92 ± 7.06%), with the L2 group having greater effects on wound contraction rate in comparison to the L1 group (Figure 2b). The above results about wound area and rates of wound contraction were shown in Figure 2c.

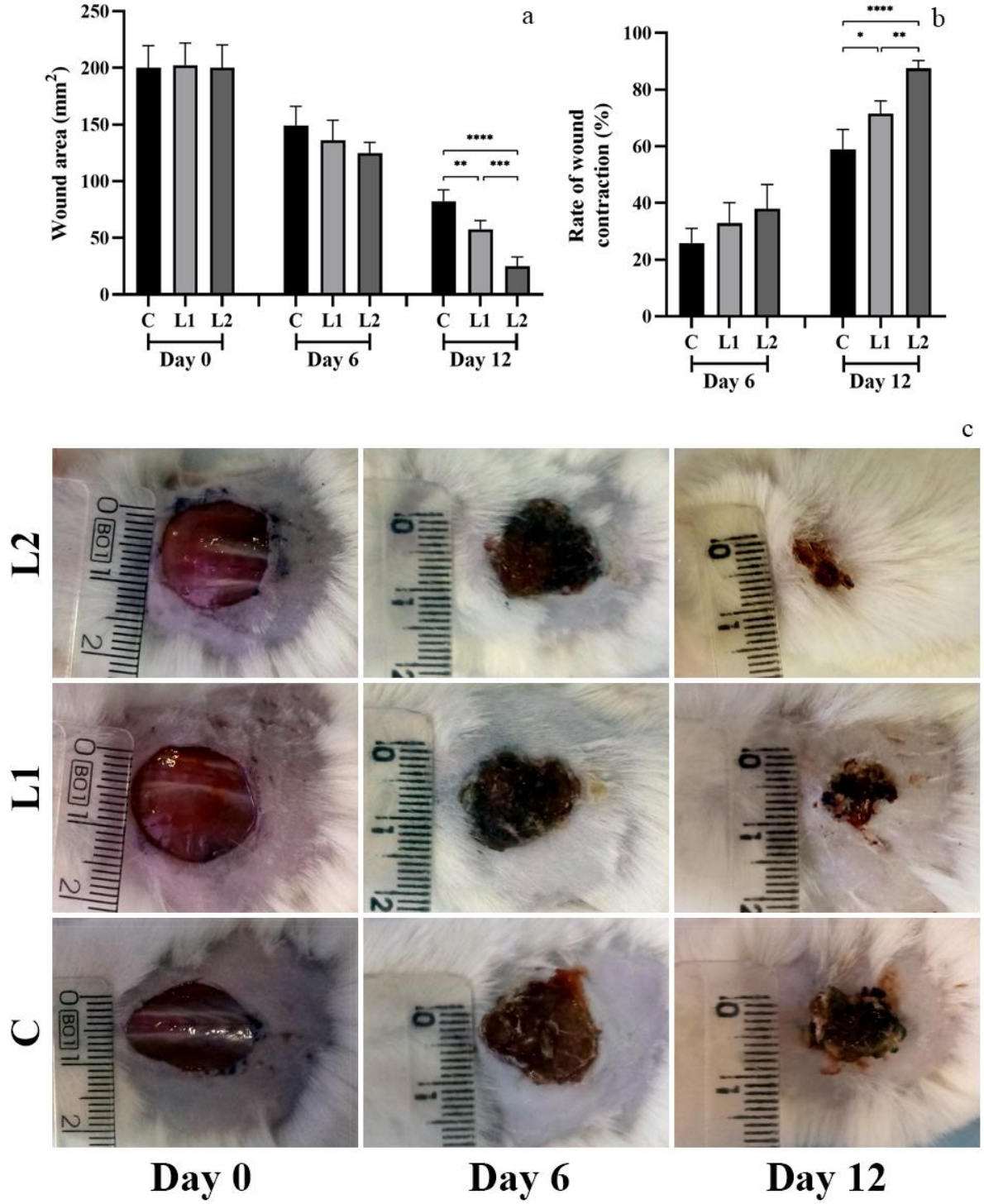

Figure 2. Evolution of the healing process of second intention wounds in mice untreated and treated with peptides of the fraction F4. (a) Wound area (mm^2). (b) Rate of wound contraction (%). Representative images of wounds in mice untreated and treated with peptides of the fraction F4 (c). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 7) are expressed as mean and standard deviation. Statistical difference between groups *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

Histopathological results

Our histopathological results showed that there was no significant difference regarding the total number of cells between the treated groups with peptides of the fraction F4 and control group throughout the experiment period (Figure 3a). The same was observed concerning the number of blood vessels (Figure 3b) and mast cells (Figure 3c).

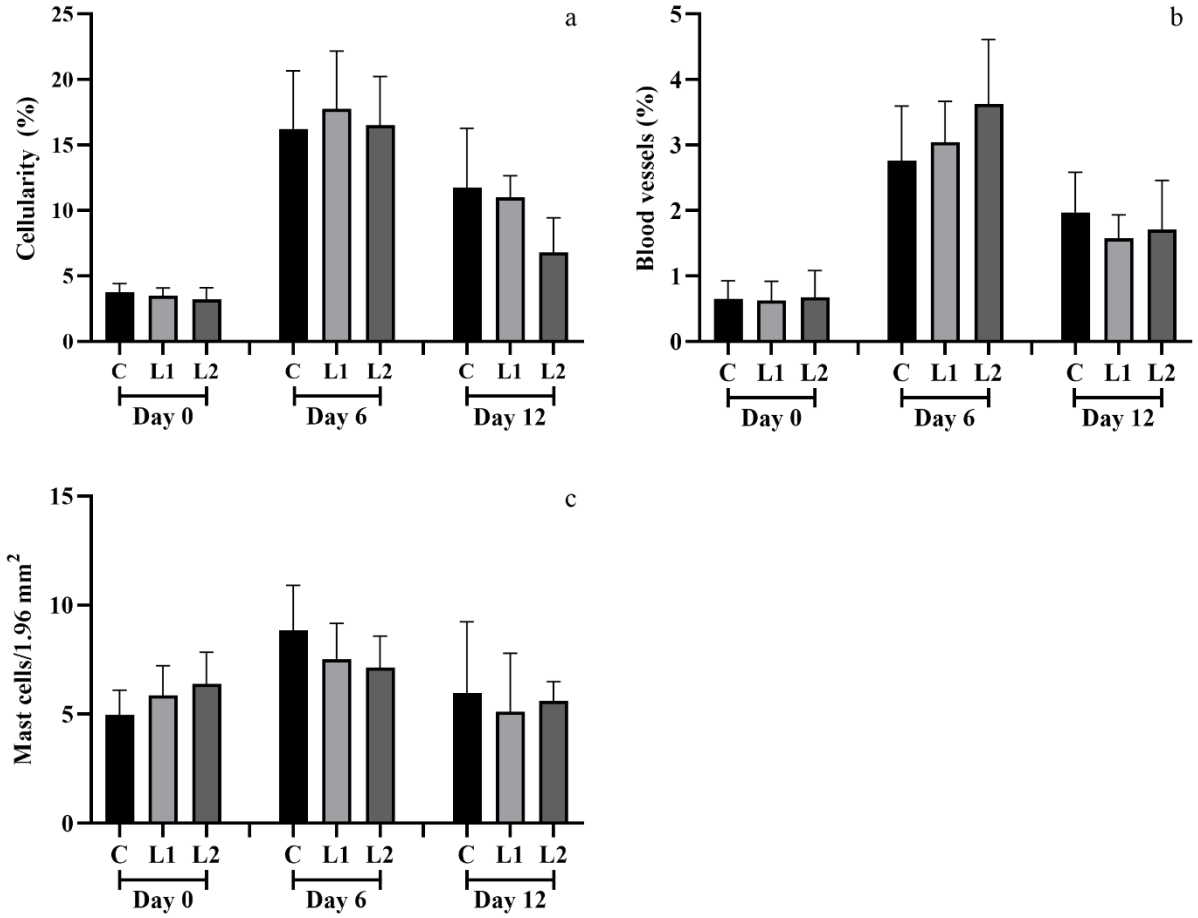

Figure 3. The proportion of cells and blood vessels in the tissue of mice untreated and treated with peptides of the fraction F4. (a) Total cells (%). (b) Blood vessels (%). Mast cells (c). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 7) are expressed as mean and standard deviation. There was no statistical difference between the groups (Kruskal-Wallis test with Dunn's test).

The proportion of type I collagen fibers was considerably higher in the L2 group compared to the control and L1 group on days 6 and 12 post-injury (Figure 4a). The type III collagen fibers were reduced in L1 and L2 groups, on day 12, in comparison to the control group (Figure 4b). Collagen deposition during the healing process can be seen in Figure 4c.

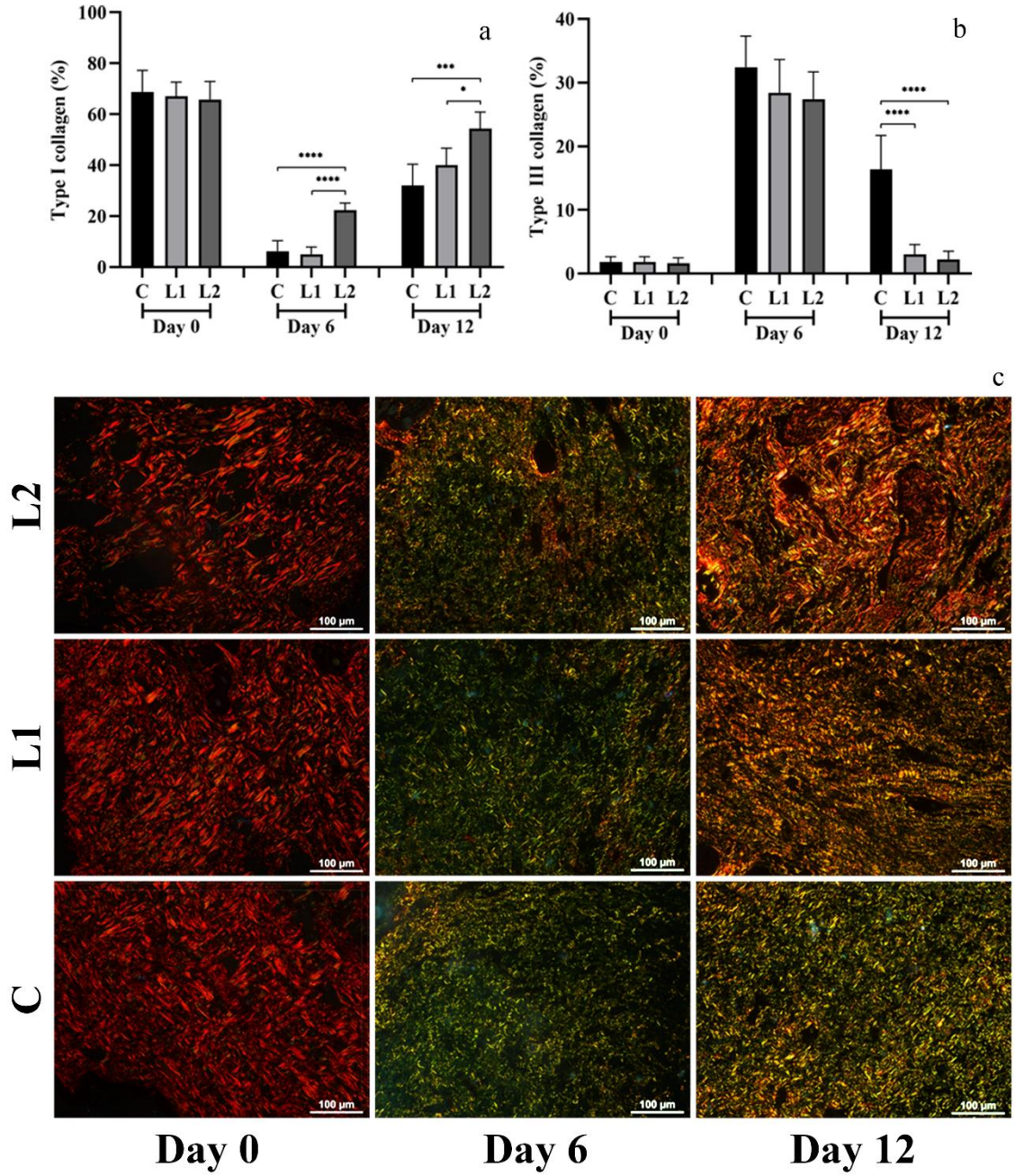

Figure 4. Deposition of collagen fibers in the tissue of mice untreated and treated with peptides of the fraction F4. (a) Type I collagen fibers (%) and (b) type III collagen fibers (%). (c) Representative photomicrographs showing collagen fibers distribution (type I collagen = red or yellow and type III collagen = green, Sirius red staining under polarized light, bar = 100 µm). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 7) are expressed as mean and standard deviation. Statistical difference between groups *p < 0.05, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

Immunofluorescence results

The results of the COX-2 analysis showed that the groups treated with peptides of the fraction F4 had significantly lower values compared to the control group on days 6 and 12 post-injury, with the L2 group showing considerably lower values in comparison to the control and L1 group (Figure 5a). The results of the F4/80 analysis showed that groups treated with peptides of the fraction F4 presented significantly lower values compared to the control group on days 6 and 12, in which L2 group had significantly lower values than L1 group (Figure 5b). In the

CD163 analysis, L2 group had significantly lower values compared to the control group on day 6, while on day 12, all groups treated with peptides of the fraction F4 presented significantly lower values compared to the control group (Figure 5c). About to Ki67 marker, groups treated with peptides of the fraction F4 showed significantly higher values compared to the control group on day 6, in which the L2 group had significantly higher values than the L1 group. While on day 12, L2 group had considerably lower values compared to the control and L1 group (Figure 5d). Photomicrographs of antibody labeling are available in Figure 6.

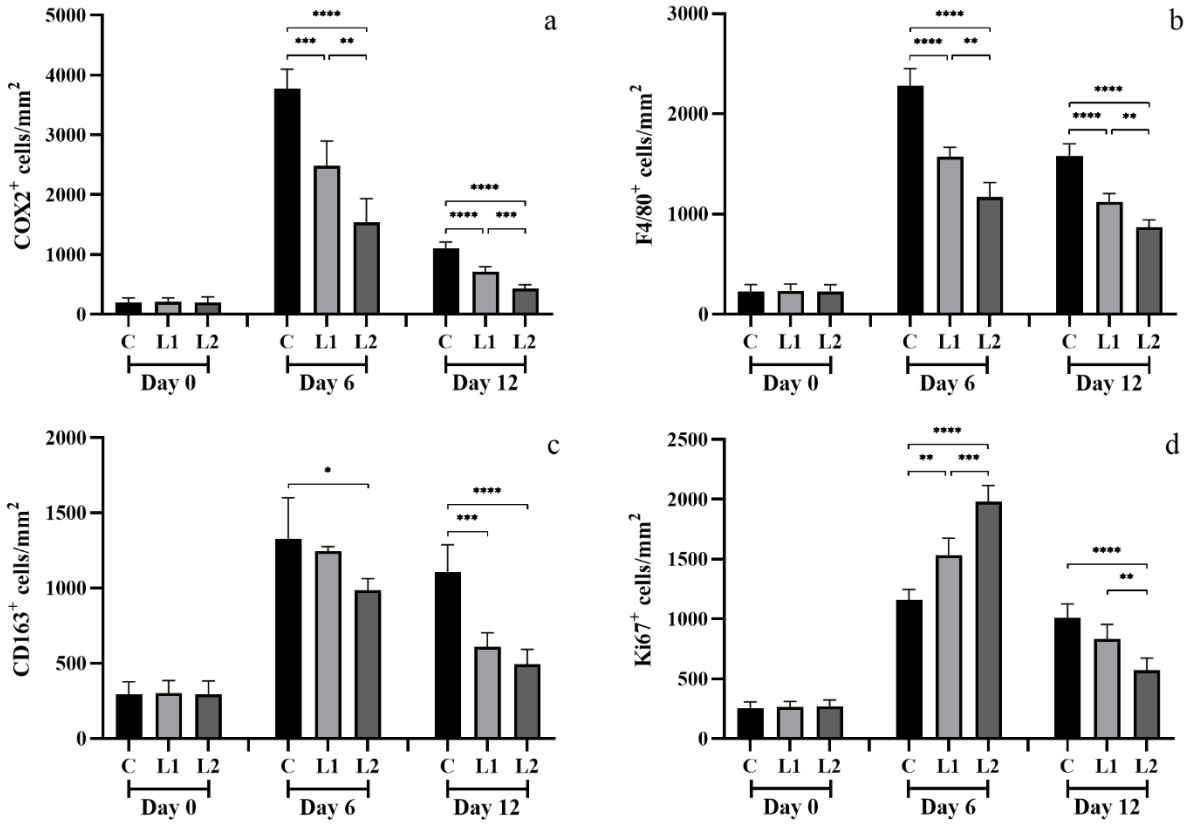

Figure 5. Antibody labeling: anti-COX-2 (a), anti-F4/80 (b), anti-CD163 (c), and anti-Ki67 (d) in the tissue of mice untreated and treated with peptides of the fraction F4. C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 5) are expressed as mean and standard deviation. Statistical difference between groups *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

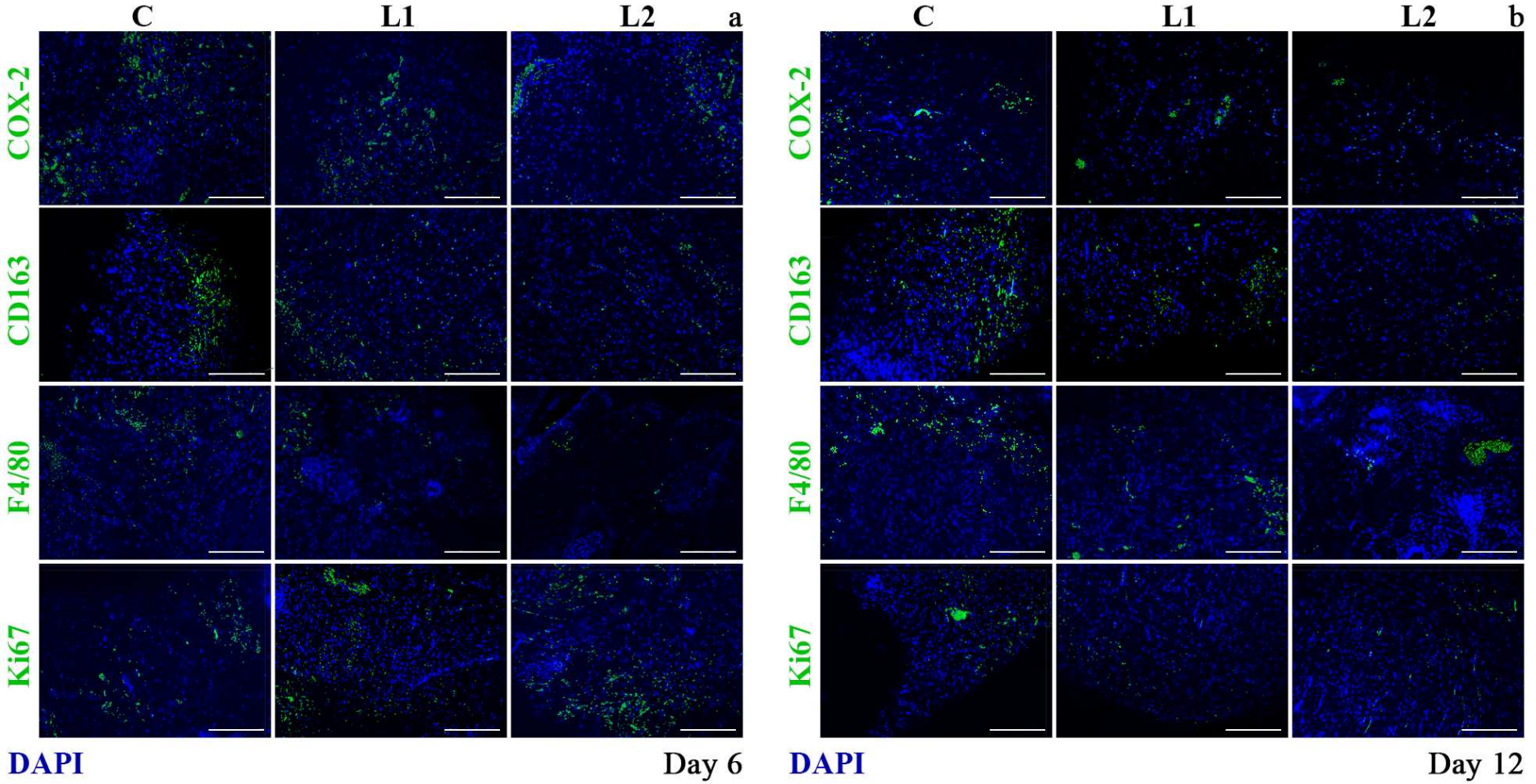

Figure 6. Immunofluorescence labeling with antibodies in green (COX-2, CD163, F4/80, and Ki67) and nuclei in blue (DAPI) in tissue from mice untreated and treated with peptides of the fraction F4 on days 6 (a) and 12 post-injury (b) (scale bar = 150 µm).

Oxidative stress markers and antioxidant enzyme activity

Malondialdehyde levels were significantly lower in the groups treated with peptides of the fraction F4 compared to the control group on days 6 and 12 post-injury (Figure 7a). Concerning carbonyl protein levels, significantly lower results were found in the groups treated with peptides of the fraction F4 compared to the control group only on day 6 (Figure 7b).

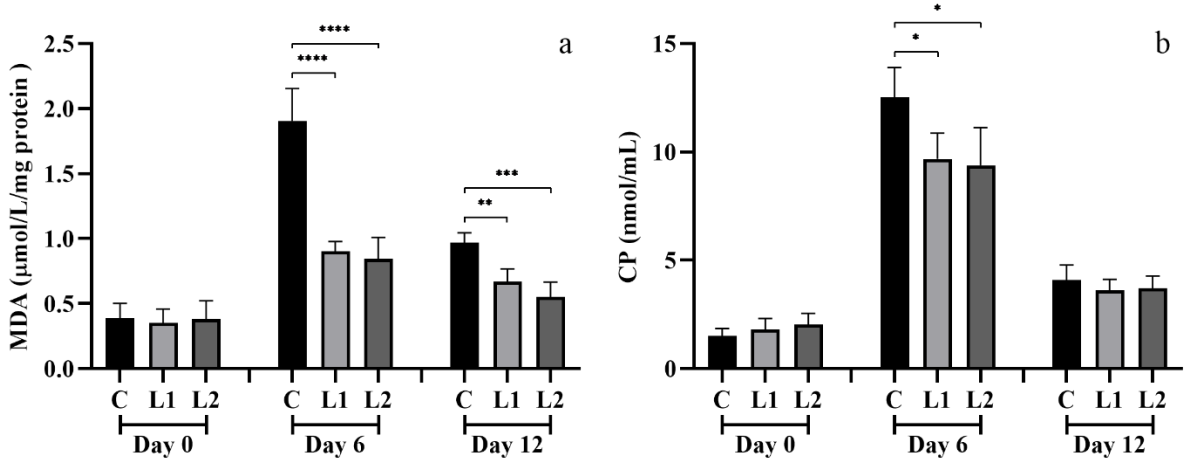

Figure 7. Levels oxidative stress markers in tissue of mice untreated and treated with peptides of the fraction F4: malondialdehyde (a) and carbonyl proteins (b). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 5) are expressed as mean and standard deviation. Statistical difference between groups *p < 0,05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

There was no significant difference in the activity of the antioxidant enzymes SOD and GST between the groups treated with peptide of the fraction F4 and control group throughout the experimental period. (Figure 8a and Figure 8b). However, significantly higher levels of CAT enzyme activity were observed in the L2 group compared to the control group on day 6 post-injury (Figure 8c).

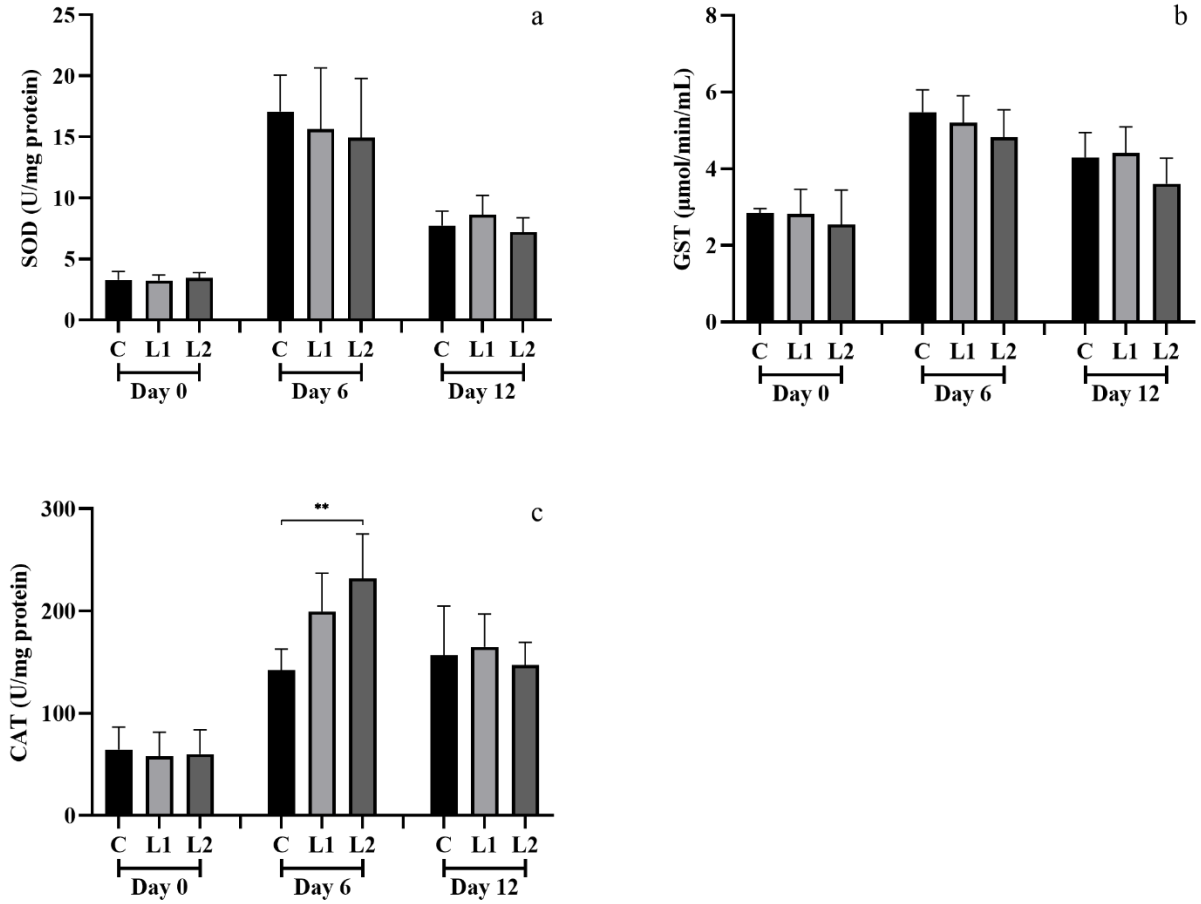

Figure 8. Activity levels of antioxidant enzymes in tissue of mice untreated and treated with peptides of the fraction F4: superoxide dismutase (a), glutathione S-transferase (b), and catalase (c). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 5) are expressed as mean and standard deviation. Statistical difference between groups **p < 0.01 (one-way ANOVA with Tukey's t-test).

Inflammation markers gene expression

The results of TLR-4 gene expression were considerably lower in the L1 and L2 groups compared to the control group on day 6 (Figure 9a). The IL-1β gene expression was considerably lower in the L2 group compared to the control group on day 6 post-injury (Figure 9b). IL-6 gene expression results were considerably lower in the groups treated with peptides of the fraction F4 compared to the control group on days 6 and 12, with the L2 group showing significantly lower values in comparison to the L1 group (Figure 9c). About COX-2, L2 group had considerably lower values compared to the control and L1 group on day 6, while on day 12, groups treated with peptides of the fraction F4 showed considerably lower values compared to the control group (Figure 9d).

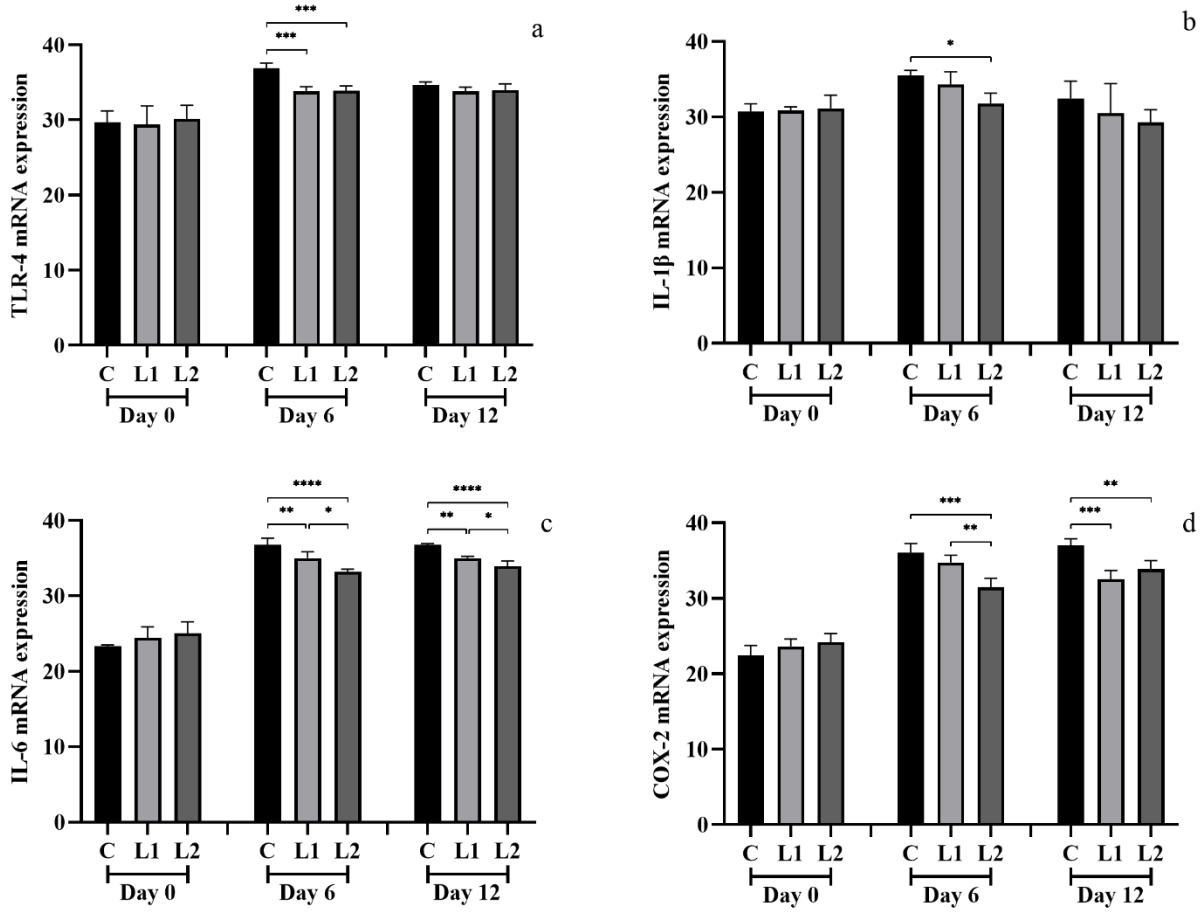

Figure 9. Effect of peptides of the fraction F4 on mRNA expression: TLR-4 (a), IL-1β (b), IL-6 (c), and COX-2 (d). C: control (distilled water), L1: peptides (3.125 µg/mL), and L2: peptides (25 µg/mL). The data (n = 5) are expressed as mean and standard deviation. Statistical difference between groups *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (one-way ANOVA with Tukey's t-test).

Discussion

Amphibians have developed over time a unique and highly efficient peptide skin defense system, composed of peptides with different characteristics and bioactivity [43]. Amphibian skin has a great peptide diversity with biological activity still little explored and many therapeutic applications [44,45]. Studies have shown the positive effects on skin wound healing using peptides isolated from amphibian skin secretions [19,20]. Recently we did a systematic review to evaluate the preclinical evidence on the impact of animal peptides on skin wound healing and observed that the compounds obtained from amphibians promote an increase in the reepithelialization, vascularization, and cell proliferation, as well as collagen deposit and consequently promoting fast wound closure [46]. In addition, was possible to observe that the enzyme hydrolysis process is another interesting way to obtain peptides since the hydrolysis of proteins from extracellular matrix proteins releases peptides with biological activities not directly exhibited by the parental protein, which includes angiogenic, antimicrobial, mitogenic, and chemotactic properties [47]. In this context, our results show that the accelerated effect of the wound closure can be related to the low molecular weight peptides present in the fraction F4. Wound area and contraction rate are the main variables evaluated in excisional wound models, as they allow the assessment of the action of potential therapeutic agents in the rapid closure of cutaneous wounds [48]. The study using low molecular weight peptides (10-15 kDa) derived from collagen from *Rhopilema esculentum* by pepsin,

demonstrated greater healing effects when compared with peptides of higher molecular weight [49]. Similar results were observed in the use of peptides from the skin secretion of *Rana ridibunda* in wound healing, where better results were observed in the use of peptides with molecular weight < 10 kDa [50]. Although animals and biological sources of peptides are different, low molecular weight peptides seem to have better effects on the healing process, however, more studies are needed to evaluate this relationship and understand the main mechanisms activated after peptides exposure.

The studies using *in vitro* analyses are a very important tool to help us to direct in our *in vivo* studies and our study was not different. The cell viability test allows evaluating the proliferative or cytotoxic effect of compounds that come in contact with cells, as well as determining the dose-response effect [51,52]. Our *in vitro* findings demonstrated that the peptides of the fraction F4 are effective in promoting the cell proliferation of macrophages and fibroblasts, which are important cells involved in the healing process. Macrophages are cells of the immune system that act at different stages of the healing process, coordinating inflammatory, proliferative, and angiogenic processes [53]. Fibroblasts are cells that after being activated in the granulation tissue express alpha-smooth muscle actin (α-SMA) and acquire the phenotype of myofibroblasts, acting in the deposition of collagen, other components of the extracellular matrix, and wound contraction [54].

After a traumatic event and cell lysis as in skin lesion, damage-associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP), deoxyribonucleic acid (DNA) fragments, fibronectins, small fragments of hyaluronan, and cytokines are released and stimulate immunomodulatory or inflammatory effects [55]. DAMPs can stimulate pattern recognition receptors on immune cells, such as formyl peptide receptor-1 (FPR1) and TLRs on tissue-resident macrophages and infiltrating neutrophils, activating the classical nuclear factor-kappa B (NF-κB) pathway [56]. When TLR-4 recognize DAMPs, the MyD88 binds to the Toll/IL-1 receptor (TIR) domain of TLRs, which triggers the intracellular signaling cascade [57]. The activation of NF-kB cascade involves a signaling complex that contains MyD88, IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF-6) [58,59]. Lastly, transcription is initiated to express proinflammatory cytokines such as IL-1β and IL-6, or other inflammatory molecules, such as nitric oxide (NO) produced by the inducible nitric oxide synthetase (iNOS), COX-2, ROS, and MMPs [60,61]. Resting macrophages produce low levels of inflammatory mediators, however, on exposure to proinflammatory cytokines, lipopolysaccharides, extracellular matrix fragments, and DAMPs, macrophages acquire the M1 phenotype [62]. M1 macrophages produce a large number of proinflammatory mediators and cytokines such as IL-1β, IL-6, and COX-2 [63]. All these factors are essential in the early stages of the inflammatory phase for the recruitment of immune system cells and the response against pathogens [64]. IL-6 can stimulate the migration of fibroblasts to the wound bed by JAK/STAT and mitogen-associated protein kinase (MAPK) signaling pathway [65]. In addition, macrophages secrete IL-1β and tumor necrosis factor-α (TNF-α) in response to IL-6 exposure, which induces the production of keratinocyte growth factor (KGF) by fibroblasts, and consequently promotes the activation of keratinocytes, stimulating proliferative and migratory effects [66–68]. COX-2 acts in the arachidonic acid degradation pathway producing prostaglandin E_2 (PGE_2), which stimulates the inflammatory process, increasing vascular permeability and the infiltration and activation of inflammatory cells [69]. However, overexpression of these mediators and cytokines in the wound bed contribute to inflammation and persistence of inflammatory cells, increasing the production of free radicals and ROS, delaying the healing process [70]. Thus, the control of inflammation by downregulation IL-1β, IL-6 and COX-2 may be a relevant strategy by which peptides of the fraction F4 decrease the infiltration of inflammatory cells in the tissue. The reduction in gene expression of these molecules may be related to the downregulation of TLR-4, since this receptor is involved in the

NF-κB activation [71]. The high expression of TLR-4 stimulates the polarization of the M1 macrophage and decreases the polarization of the M2 macrophage [71]. Macrophage polarization from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype is essential for the progression of the healing process, which is mediated by a set of specific factors, including efferocytosis of apoptotic cells present at the site of inflammation, cytokines, lipid mediators, and other cell signaling mediators [72]. M2 macrophage are stimulated by interleukin-4 and interleukin-13 and express high levels of the interleukin-10, transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), which are important cells for fibroblast proliferation, matrix deposition, angiogenesis and tissue repair [53]. In our study, we observed that the peptides of the fraction F4 decreased the number of total macrophages and COX-2 in scar tissue. Interestingly, the number of M2 macrophages was also reduced, which may be related to the reduced number of total macrophages. Some studies suggest that it is the same macrophages that regulate early inflammatory and later wound repair functions [72,73]. Since peptides of the fraction F4 are involved in resolving the inflammatory phase, potentially attenuate oxidative stress caused during a prolonged inflammatory phase.

In skin lesions, the synthesis of free radicals and ROS by inflammatory cells contributes to the defense against pathogens and mediates important intracellular pathways for the resolution of the inflammatory phase [74]. However, excessive amounts of free radicals and ROS promote cellular oxidative stress, causing deleterious effects on cell membranes, proteins, and nucleic acids of cells involved in the repair process, leading to tissue damage [17]. The oxidative stress markers are important tools to understand the effect of these compounds inside the cells and have been contributes to understanding the damage caused by these stressors, through the indirect analysis of oxidation products of lipids, proteins, and nucleic acids [75]. In this sense, compounds generate through lipid and protein oxidation like malondialdehyde and carbonyl proteins are considered important oxidative stress markers used in wound healing analyses and are intimately related to the inflammatory process [17]. In our study, we observed reduction in the MDA and CP levels, indicating that peptides of the fraction F4 can help to positively modulate the redox state of the tissue in recovery. It is important also to understand those inflammatory ways that are involved in oxidative stress in damaged tissue as Nrf2 pathway, which is always activated under conditions of oxidative stress [76]. Nrf2 is a key transcription factor in regulating the expression of antioxidant response genes, encoding for antioxidant enzymes in various types of cells and tissues [77]. Regulation of this pathway is involved in the production of CAT and GST enzymes, but not SOD [78], which is regulated by other pathways such as PI3K/Akt and NF-κB [79]. Thinking in this relationship, we evaluated the activity of these three antioxidant enzymes to understand how peptides may be influencing the antioxidant defense system in scar tissue. SOD is responsible for the dismutation of the superoxide radical into hydrogen peroxide and water [80]. Although hydrogen peroxide is not radical, it can cause severe cell damage due to the generation of hydroxyl radicals in the presence of iron or copper ions (Fenton reaction) [17]. CAT is responsible for converting hydrogen peroxide into molecular oxygen and water, reducing the risk of cell damage [81]. GST is responsible for catalyzing the conjugation of GSH with several compounds that are produced during oxidative stress [82]. Therefore, the antioxidant potential of a compound can be analyzed by its capacity to increase the activity of these enzymes. Our results showed that our compound was effective in promoting the increase of catalase activity, accelerating the passage of the third electron, and delaying the contact time of free radicals with components inside the cells. These data suggest better redox control in the scar tissue in the groups treated with peptides of the fraction F4, mainly through the action of the catalase enzyme, highlighting the importance of the redox balance for wound healing.

In our histological analysis, there were not many differences regarding cellularity, blood vessels, and mast cells. However, when analyzing the cell proliferation marker (Ki67) by

immunofluorescence, we observed that there was an increase in the quantity expression of this marker, indicating that there was an increase in cell proliferation after 6 days of exposure to the peptides of the fraction F4. This time is coinciding with the transition between the inflammatory and proliferative phase of the wound healing process, in which cell proliferation is crucial for the good development of the regenerative process [83]. These findings justify us *in vitro* results that also showed that peptides of the fraction F4 are good for promoting cell proliferation to fibroblasts. In addition, the decline in Ki67 expression in the late phase of the healing process may be related to the transition from the proliferative phase to the tissue remodeling phase [84], which was evident in this study, by the decrease in Ki67 labeling and increase in the proportion of type I collagen fibers on day 12. Similar results were found in the study of wound healing using the cathelicidin-NV peptide, where an increase in fibroblast proliferation *in vitro* and greater collagen deposition in scar tissue was observed [20]. We observed that peptides of the fraction F4 stimulate an increase in the proportion of type I collagen fibers, associated with a low proportion of type III collagen fibers, after 12 days of treatment. Type III is found in greater proportions early in the healing process, but as the healing process progresses, type III collagen are replaced by type I collagen [85]. These characteristics are very important for increasing the strength of scar tissue, as type I collagen fibers are thicker and make the tissue more resistant to traction [86]. Our histological results suggest that the use of peptides of the fraction F4 obtained from enzymatic hydrolysis of bullfrog skin by trypsin can promote the formation of a more resistant granulation tissue by increasing type I collagen fibers in the scar tissue and promoting wound closure.

Conclusion

Taken together, our findings indicate that the peptides of the fraction F4 obtained from enzymatic hydrolysis of bullfrog skin by trypsin can modulate the repair of skin wounds in mice, with the best results observed after exposure to the highest concentration tested (25 μg/ml). We observed that the peptide profile of the fraction F4 was composed of short peptide sequences of low molecular weight. *In vitro*, our findings showed that peptides of the fraction F4 increased macrophage and fibroblast proliferation. Our *in vivo* findings indicate that the downregulation of TLR-4, IL-1β, IL-6 and COX-2 induced by the peptides of the fraction F4 can reduce the inflammatory response, and this capacity of modulating the intensity of the inflammatory response and promote cell proliferation can play an important role in the wound healing process. The morphological consequences for the exposure of the investigated peptide fraction were the reduction of the inflammatory process and oxidative stress, along with increased cell proliferation, maturation of the extracellular matrix and faster wound closure. However, further studies are needed to understand the others mechanisms involved in the wound healing process and its relationship with inflammatory markers and oxidative markers during the skin regenerative process.

Conflicts of interest

No competing financial interests exist.

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Supplementary material

S1 Table. Protein-related peptide sequences found in the hydrolyzed extract of bullfrog skin by trypsin.

S2 Table. Protein-related peptide sequences found in the hydrolyzed extract of bullfrog skin by trypsin using BLAST software.

S1 Figure. Flowchart of experimental processes.

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S1 Table. Protein-related peptide sequences found in the hydrolyzed extract of bullfrog skin by trypsin.

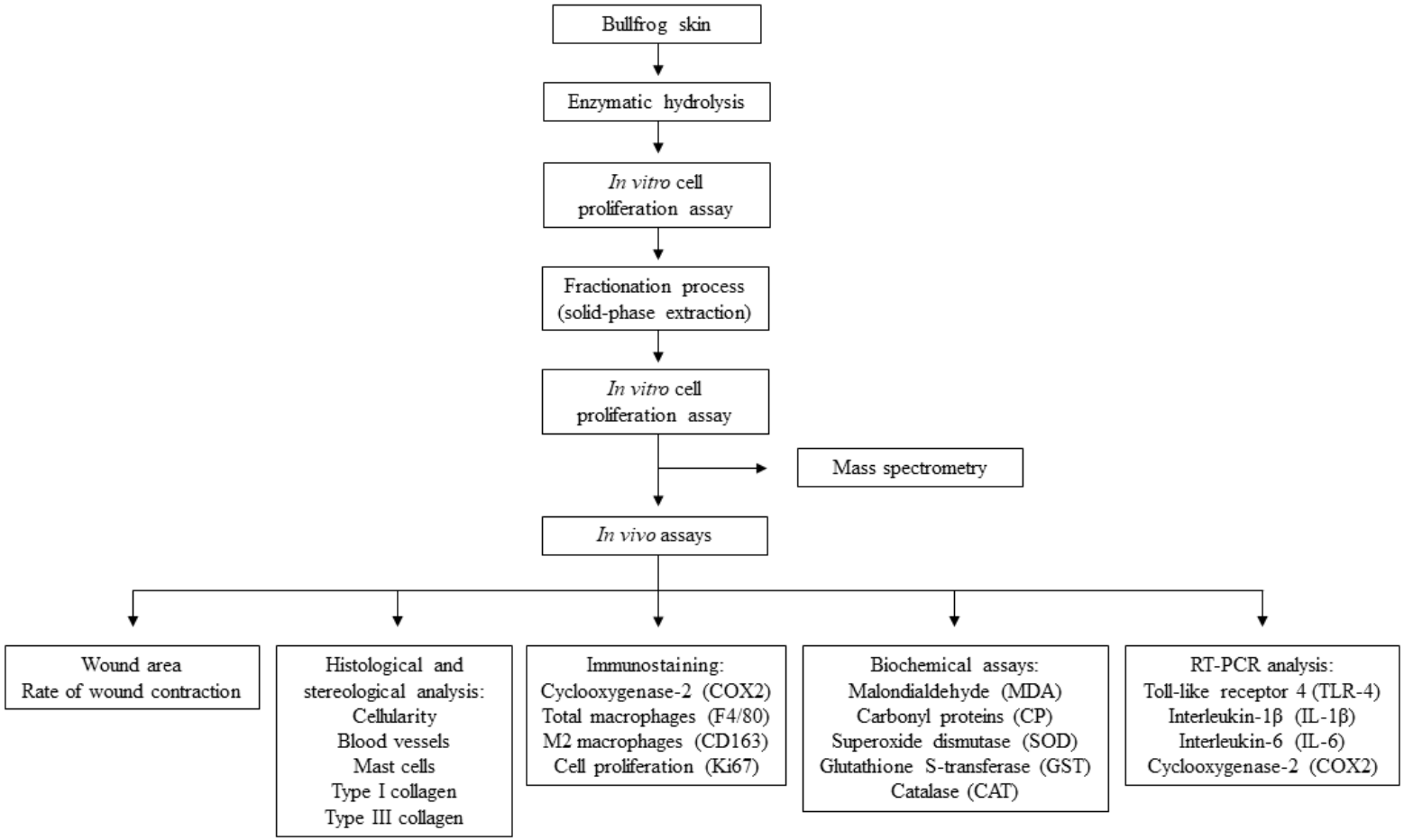
Protein accession	Protein description	Peptides	Mass (Da)
A0A2G9S0U7	Collagen alpha-2 (I) chain	SGNRGEGGPSGPAGITGPSGPRGPAGPQGVR	2783.3811
		SGHPGAMGPVGPR	1218.5928
		GPSGPSGPPGKEGR	1278.6316
		GAPGERGEAGPAGPTGFAGPPGAAGHTGAKGDR	2927.4021
		IGPAGSAGSR	871.4512
		AGGIGPAGSR	841.4406
		GIPGPAGPAGPSGAR	1260.6575
		GPAGAAGAPGPAGGPGDRGESGPAGPSGVAGPR	2708.3013
		SLNQQIEVILTPEGSRK	1911.0425
A0A2G9SH44	Collagen alpha-1 (I) chain	GLTGPIGPPGPGGAPGDKGEAGPAGPAGPTGSR	2806.3997
		GPAGPPGSTGFPGAAGR	1452.7109
		SAGISMPGPMGPMGPR	1541.7152
		GPPGPSGPPGLAGPPGEPGR	1749.8798
		GPSGPAGARGDK	1068.5311
		GQSGVMGFPGPK	1176.5597
		NGDRGETGPAGPAGPAGPAGAR	1931.9197
A0A2G9QE21	Hemoglobin subunit beta	VLNSIEEGLKHPENLK	1818.9839
		LLIVYPWTQR	1287.7339
		LGDVLIVTMAR	1186.6743
		VLNSIEEGLK	1100.6077
		GGSDVSAFLAK	1050.5345
		HSGELHVDPANFYR	1640.7695
Q8QGD4	Monomeric alpha-macroglobulin	KGDSTVVIEDDSGFHR	1760.8329
		RLDEHATIEGETK	1497.7423
		QFIHVDDQDIQK	1484.726
		DAQTKFDVHIEAR	1528.7633
A0A2G9R467	Actin cytoplasmatic 2	SYELPDGQVITIGNER	1789.8846
		VAPEEHPVLLTEAPLNPK	1953.057
		QEYDESGPSIVHR	1515.6953
		AVFPSIVGRPR	1197.6981
P21847	Albumin	LLFLAHFTHDYAR	1602.8307
		EFPDIVFK	993.5171
		MPQVTAPTLVELAGR	1581.8549
		GLTLVQVSQKFGK	1403.8136
A0A385KNQ9	Vimentin-4	VEVDRDNMADDLQR	1674.7631
		FADLSEAANR	1092.5199
		KLESLQEEIIFLK	1588.9076
A5LHA5	Annexin	DLVDDLKSELTGK	1431.7456
		VNDALVEQDAQDLFK	1703.8365
		GLGTDEDPIMK	1174.554
C1C502	Tropomyosin-1 alpha	IQLVEEELDR	1242.6455
		HIAEEADRKYEEVAR	1814.8911
		LVIIEGDLER	1155.6499
A0A2G9RE03	2-phospho-D-glycerate hydro-lyase	AAVPSGASTGIYEALELRDNDKTR	2533.2771
		QIFDSRGNPTVEVDLFTAK	2136.085
C1C500	Histone H4	TVTAMDVVYALKR	1465.7963
		DNIQGITKPAIR	1324.7462
		VFLENVIR	988.5706
A0A2G9SLW0	IF rod domain	DLSLDGILR	1000.5553
		LADLEAALQK	1070.5972
A0A2G9SAK4	Fibrinogen C-terminal	LIGTKPATSPQVIENPTQK	2021.1157
		ESVQIQEITGK	1230.6455
A0A2G9QD88	AhpC-TSA	QITINDLPVGR	1224.6826
		GLFIIDEKGILR	1372.8077

P55267	Hemoglobin subunit alpha	VMNALTDAVK	1060.5587
		TYFPNFDFHANSAHLK	1907.8954

IF: intermediate filament.

S2 Table. Protein-related peptide sequences found in the hydrolyzed extract of bullfrog skin by trypsin using BLAST software.

Protein accession	NCBI accession	Protein description	Max score	Total score	Query cover	Per. ident	Peptides	Mass (Da)
A0A2G9NCH1	XP_040213459	Collagen alpha-3 (VI) chain isoform X6	4403	6667	96%	93.98%	SAGSRIEEQVPQHLVLLTGGKSVDDVSGAAR	3175.6584
							VSELNTGDIEILR	1457.7726
							SIQSMTPIGGSTLNTGAALDYVQNNVFIGSAGSR	3425.6885
							DVVFLIDGSRDATPEFANVKELIGR	2760.4446
							SSDNIQAAANDLIR	1486.7375
							LLSSVTNLDQDSIKVIYENVPR	2502.333
							IGTGVPQIAFIITGAK	1584.9238
							SSGVIPFAIGVR	1201.6819
							ELPNIESILFR	1329.7292
							VVEALDVDRDKIR	1526.8416
							VGLVQFSNDPTTEFFLK	1940.9883
A0A2G9P807	XP_040205899	Collagen alpha-1 (XII) chain isoform X3	3239	4803	98%	96.41%	LVEVFEIGPER	1286.687
							NLNIYDIGTTTMR	1510.745
A0A2G9RV90	XP_040213464	Collagen alpha-3 (VI) chain isoform X11	704	1953	100%	97.50%	IVNRLEIGPDLIR	1506.8882
							ESVQIQEITGK	1230.6455



S1 Figure. Flowchart of experimental processes.

4. CONCLUSÕES GERAIS

A partir de estudos utilizando modelos pré-clínicos, identificamos que diversos peptídeos de origem animal são eficientes em promover a aceleração do reparo cutâneo, por efeitos relacionados a proliferação celular, neoangiogênese, colagenogênese, reepitelização e contração da ferida. Contudo, ficou evidente a fragilidade dos estudos existentes na literatura sobre o tratamento de feridas cutâneas utilizando peptídeos de origem animal, uma vez que a maioria dos artigos incluídos na revisão sistemática apresentou alto risco de viés.

O uso peptídeos obtidos por meio da hidrolise enzimática da pele de rã-touro demonstrou modular o reparo de feridas, favorecendo a transição da fase proliferativa para a fase de remodelação tecidual, por meio do aumento da atividade proliferativa das células. Além disso, a administração destes peptídeos também aponta para uma redução da resposta inflamatória e do dano oxidativo, resultando no fechamento acelerado da ferida.