LUCIANA SCHULTHAIS ALTOÉ

EFEITO DO USO DE ANTIBIÓTICOS NO PROCESSO DE REPARO CUTÂNEO EM MODELO ANIMAL

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: Rômulo Dias Novaes
Mariáurea M. Sarandy Souza
Sirlene S. Rodrigues Sartori

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Luciana S. Altoé

Luciana Schulthais Altoé
(Autora)

Reggiani Vilela Gonçalves
(Orientadora)

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RESUMO

ALTOÉ, Luciana Schulthais, D.Sc., Universidade Federal de Viçosa, julho de 2019. **Efeito do uso de antibióticos no processo de reparo cutâneo em modelo animal**. Orientadora: Reggiani Vilela Gonçalves. Coorientadores: Rômulo Dias Novaes, Mariáurea Matias Sarandy Souza e Sirlene Souza Rodrigues Sartori.

A busca por um tratamento eficaz que promova o fechamento rápido de feridas cutâneas tem como objetivo garantir ao paciente menor sofrimento e reduzir custos. Uma ferida pode demorar a cicatrizar devido a falha na progressão entre as fases da cicatrização. A cronificação do processo geralmente está associada a uma fase inflamatória prolongada. O objetivo desse trabalho foi investigar o uso de antibióticos, em especial da doxiciclina (Dx), na cicatrização de feridas cutâneas não infectadas em animais. Primeiro, realizamos uma revisão sistemática seguindo as diretrizes PRISMA. A plataforma SYRCLE's Risk of Bias foi utilizada para análise da qualidade metodológica dos estudos. Em nossa revisão, vinte e sete estudos foram selecionados para a extração de dados e análise de viés metodológico. A compilação dos dados mostrou que o tratamento antibiótico pode promover redução do infiltrado inflamatório, aumento do número de fibroblastos e dos constituintes da matriz extracelular, reepitelização e aumento resistência mecânica do tecido neoformado. No entanto, observamos grande viés metodológico entre os estudos, o que compromete a validade interna e externa dos mesmos. Após os achados promissores encontrados nesta revisão, partimos para investigação da ação do antibiótico doxiciclina, in vitro na viabilidade celular de macrófagos e in vivo no reparo cutâneo. Ratos Wistar machos (n=15) foram alojados em gaiolas individuais com comida e água ad libitum (número de registo 72/2017). Após anestesia, três feridas circulares (12 mm de diâmetro) foram feitas no dorso de cada animal. Estes foram divididos em três grupos e receberam o tratamento durante 21 dias, via gavagem: C (controle - água); Dx1 (Dx 10 mg/kg/dia) e Dx2 (Dx 30 mg/kg/dia). A área e a taxa de contração das feridas foram avaliadas e os tecidos neoformados foram coletadas para análise histologia e bioquímica. Animais tratados com Dx apresentaram maior proporção de células (macrófagos e mastócitos) e dos componentes da matriz extracelular (colágeno e fibra elástica), aumento inicial seguido de redução a proporção de vasos sanguíneos e reduziu o estresse oxidativo e os níveis de metaloproteinase de matriz-10 (MMP-10) do tecido cicatricial. Além disso, resultou em maior contração da ferida e melhor cicatrização em relação ao controle. Em conclusão, os antibióticos, que são drogas com ampla utilização na população, podem interferir no reparo de feridas

cutâneas não decorrente de infecção, evidenciando a eficácia do antibiótico Dx no processo de cicatrização de feridas de segunda intenção em ratos Wistar.

Palavras-chave: Antibioticoterapia. Revisão sistemática. In vitro e in vivo análises. Cicatrização de ferida.

ABSTRACT

ALTOÉ, Luciana Schulthais, D.Sc., Universidade Federal de Viçosa, July, 2019. **Effect of the use of antibiotics on the skin repair process in animal model**. Advisor: Reggiani Vilela Gonçalves. Co-advisors: Rômulo Dias Novaes, Mariáurea Matias Sarandy Souza and Sirlene Souza Rodrigues Sartori.

The search for an effective treatment that promotes rapid closure of skin wounds aims to ensure the patient less suffering and reduce costs. A wound may take time to heal due to failure to progress between healing phases. Chronification of the process is usually associated with a prolonged inflammatory phase. The aim of this study was to investigate the use of antibiotics, especially doxycycline (Dx), in the healing of uninfected skin wounds in animals. First, we performed a systematic review following the PRISMA guidelines. The SYRCLE's Risk of Bias platform was used to analyze the methodological quality of the studies. In our review, twenty-seven studies were selected for data extraction and methodological bias analysis. The compilation of data showed that antibiotic treatment can promote reduction of inflammatory infiltrate, increase in the number of fibroblasts and extracellular matrix constituents, reepithelization and increased mechanical resistance of neoformed tissue. However, we observed a great methodological bias between the studies, which compromises their internal and external validity. After the promising findings found in this review, we set out to investigate the action of doxycycline antibiotic, in vitro on macrophage cell viability and in vivo on skin repair. Male Wistar rats (n = 15) were housed in individual cages with food and water ad libitum (registration number 72/2017). After anesthesia, three circular wounds (12 mm in diameter) were made on the back of each animal. These were divided into three groups and received treatment for 21 days via gavage: C (control - water); Dx1 (Dx 10 mg / kg / day) and Dx2 (Dx 30 mg / kg / day). Area and rate of wound contraction were evaluated, and newly formed tissues were collected for histological and biochemical analysis. Dx-treated animals had a higher proportion of cells (macrophages and mast cells) and extracellular matrix components (collagen and elastic fiber), an initial increase followed by a reduction in the proportion of blood vessels and reduced oxidative stress and matrix-metalloproteinase levels. 10 (MMP-10) of scar tissue. In addition, it resulted in greater wound contraction and better healing compared to control. In conclusion, antibiotics, which are widely used drugs in the population, may interfere with the repair of non-infection skin wounds, showing the efficacy of the antibiotic Dx in the healing process of secondary intention wounds in Wistar rats.

Keywords: Antibiotic therapy. Systematic review. In vitro and in vivo analyzes. Wound healing.

SUMÁRIO

1 INTRODUÇÃO GERAL

A pele é o maior órgão do corpo e desempenha um papel fundamental como barreira entre o organismo e o ambiente externo. Ela é a primeira linha de defesa contra microrganismos patogênicos e impede ataques químicos e físicos, bem como a perda não regulada de água e solutos (MARROT, 2017). Além disso, participa de processos metabólicos como síntese de vitamina D, além de desempenhar papel sensorial e de termorregulação (MCLAFFERTY et al., 2012).

A pele é composta por duas camadas principais: a epiderme (camada externa) e a derme (camada mais interna) (MCLAFFERTY et al., 2012). A epiderme é composta por epitélio escamoso estratificado queratinizado (JUNQUEIRA; CARNEIRO, 2017) e compreende camadas diferentes de células, formadas predominantemente por queratinócitos (LOSQUADRO, 2017). Trata-se de um tecido avascular que depende dos vasos sanguíneos da derme para oxigenação, fornecimento de nutrientes e remoção de resíduos metabólicos (RITTIÉ, 2016). A derme é uma camada de tecido conjuntivo, composta por células, em especial fibroblastos, bem como matriz extacelular (MEC) rica em colágeno e fibras elásticas. O colágeno e a elastina estão dispostos em uma rede de fibras que fornecem resistência mecânica à derme, proporcionando ao tecido força e capacidade de alongar e retrair (LOSQUADRO, 2017).

No entanto, a estrutura e as funções desempenhadas pela pele podem ser afetadas por diferentes agentes agressores como forças mecânicas e doenças metabólicas, que podem expor o tecido subcutâneo e comprometer a reparação tecidual, favorecendo a colonização e proliferação microbiana (DAI et al., 2011). Após a lesão, a pele sofre um processo complexo de reparo que envolve a migração de células sanguíneas e a proliferação de células do parênquima, além da secreção de fatores de crescimento, citocinas e proteínas da MEC. Quando todos este eventos ocorrem de forma coordenada a homeostase do tecido cutâneo e, consequentemente, do organismo é reestabelecida (SARANDY et al., 2018).

O processo de cicatrização cutânea pode ser dividido em quatro fases sobrepostas: hemostasia, inflamação, proliferação e remodelação (ORYAN et al., 2016). Na primeira fase, mediadores químicos são liberados com o objetivo de reduzir a perda sanguínea e plaquetas são recrutadas e ativa a cascata de coagulação, estabelecendo o fechamento temporário da ferida (OPNEJA et al., 2019). Após a vasoconstrição inicial, há dilatação dos vasos e aumento da permeabilidade capilar, favorecendo a perfusão nutritiva da ferida e a chegada de células imunitárias na área da lesão. A próxima fase, conhecida como inflamatória, é caracterizada pelo

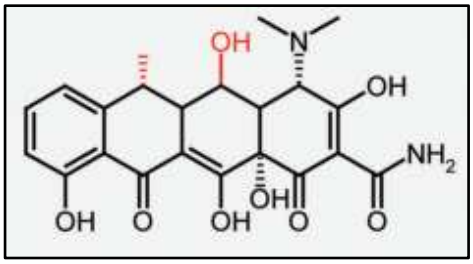
influxo de células de defesa para a área da ferida, especialmente neutrófilos e macrófagos. Essas células fazem fagocitose e liberam mediadores químicos ativando a explosão respiratória que dá origem a formação da maior parte de espécies reativas de oxigênio e nitrogênio. As citocinas pró-inflamatórias continuamente liberadas pelas células de defesa amplificam a resposta inflamatória, induzindo ainda mais a migração e proliferação celular (GONZALEZ et al., 2016) (RIDIANDRIES et al., 2018). Durante a fase proliferativa, ocorre a formação do tecido de granulação, caracterizado por grande proliferação de fibroblastos, pela síntese de fibras colágenas tipo III, fibras elásticas e formação de novos vasos (GONZALEZ et al., 2016). Este tecido é frágil e pouco resistente a trações, mas é a base necessária para a deposição do tecido definitivo (NOVAES et al., 2015). Finalmente, ocorre a fase de remodelação que é caracterizada pela contração do tecido neoformado e reorganização da MEC (XUE; JACKSON, 2015). Nesta fase, a maior parte das fibras colágenas tipo III é substituída por colágeno do tipo I, muito forte devido a capacidade de formar feixes espessos em resposta à presença de pontes cruzadas entre as fibrilas de colágeno (DESMOULIERE et al., 2014). Além disso, nessa fase ocorre apoptose ou migração da maioria das células que se originaram na fase proliferativa, especialmente miofibroblastos, macrófagos e células endoteliais. Assim, na fase de remodelação o tecido neoformado é caracterizado por predomínio de fibras colágenas e conteúdo de células, assemelhando-se à derme da pele íntegra (SABINO; KELLER, 2015; (GONZALEZ et al., 2016).

Quando a ferida não progride através das fases da cicatrização, ela pode se tornar crônica (GONZALEZ et al., 2016), causando ao paciente desconforto significativo, podendo evoluir para quadros mais graves como infecção e amputação segmentar, sepse e até mesmo morte (WHITTAM et al., 2016). Associados a estas alterações existem também elevados custos para os sistemas de saúde. Só nos EUA, avaliando-se o conjunto de dados do Medicare sobre todas as categorias de feridas agudas e crônicas, estimou que os gastos anuais no tratamento variaram entre US $ 28,1 bilhões a US $ 96,8 bilhões no período de um ano (NUSSBAUM et al., 2018). Assim, levando em consideração o impacto econômico causado por feridas e o crescimento da população de alto risco, incluindo diabéticos e idosos (LINDLEY et al., 2016) (POP; ALMQUIST, 2017), feridas podem ser consideradas um problema de saúde pública. Portanto, há uma constante busca por terapias mais eficientes que promovam o fechamento eficaz de feridas em um reduzido tempo (DHIVYA et al., 2015).

Uma das abordagens terapêuticas é o tratamento e prevenção de infecção, já que a instalação destas é um conhecido fator de impedimento para uma cicatrização rápida da ferida (HAN; CEILLEY, 2017). As bactérias são os agentes microbianos que mais frequentemente

infectam áreas com feridas de pele (JOHNSON et al., 2018). Assim, diferentes tipos de antibióticos de uso tópico ou oral são empregados no tratamento dessas infecções (PULIDO-CEJUDO et al., 2017). Entretanto, ainda não estão claros o impacto da antibioticoterapia sobre os mecanismos envolvidos nos processos de reparo tecidual. Acredita-se que além do controle da proliferação microbiana, alguns antibióticos podem favorecer o fechamento rápido da ferida, por favorecer a síntese de MEC. No entanto, os trabalhos pré-clínicos são fragmentados e apresentam elevado viés metodológico. Além disto, um número limitado de classes de antibióticos foi testado até o momento com objetivo de analisar o impacto destes fármacos em feridas cutâneas não infectadas. Entre eles podemos citar a doxiciclina (Figura 1), um antibiótico pertencente a classe das tetraciclinas frequentemente usado no tratamento de doenças infecciosas e não infecciosas (CHUKWUDI, 2016).

Figura 1– Estrutura química da doxiciclina



Fonte: adaptado de NGUYEN et al., 2014.

A estrutura básica entre os membros da classe das tetraciclinas é a presença de quatro anéis aromáticos, motivo pelo qual recebe o nome de “tetraciclinas” (STEPHENS et al., 1952). Suas propriedades antimicrobianas são resultado da inibição da síntese proteica por meio da ligação do fármaco ao ribossomo bacteriano (SCHÄFER, 2017) (MOORE et al., 2018).

A doxiciclina mostrou-se eficaz em inibir a liberação de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-α), IL-6 e IL-8 em cultura de queratinócitos humanos imortalizados (HaCaT) (DI CAPRIO et al., 2015). Além de inibir metaloproteinases da matriz (MMPs) (SCHÄFER, 2017), que são uma família de endopeptidases, capazes de degradar várias proteínas da matriz extracelular, remodelando-a (CALEY et al., 2015). A Dx age por meio da quelação de íon de zinco (Zn^{2+}) que é essencial para a atividade catalítica das MMPs (CASTRO et al., 2011). Além disto, a Dx tem elevado potencial no tratamento de uma variedade de distúrbios, incluindo aneurismas da aorta abdominal (BAXTER et al., 2002), fibrose pulmonar (FUJITA et al., 2006), sífilis (DAI et al., 2016) e doenças periodontais

(SPASOVSKI et al., 2016). Porém, apesar de promissor, pouco se sabe sobre a ação da doxiciclina no reparo de lesões cutâneo.

Diante do exposto, a presente tese teve como objetivo avaliar o efeito da antibioticoterapia no processo de reparo de feridas cutâneas em modelos animais. A tese foi dividida em dois capítulos que foram escritos no formato de artigo científico. No primeiro artigo é apresentada uma revisão sistemática (RS). Esse tipo de revisão possibilita avaliar, de maneira imparcial, os estudos incluídos, além disso, permite reprodutibilidade devido utilizar métodos sistemáticos, explícitos e padronizados durante todo processo de revisão (JAHAN et al., 2016). Assim, por meio de uma RS objetivou-se avaliar o efeito de antibióticos no reparo de lesões cutâneas em modelos animais. Além de investigar se o os antibióticos auxiliam ou não no processo de reparo de lesões de pele. Essa RS também foi utilizada como uma ferramenta para sintetizar a evidência referente aos tipos de antibióticos testados, as características dos modelos pré-clínicos utilizados e os protocolos de antibioticoterapia administrados. O segundo artigo trata-se de um estudo experimental, no qual foi avaliado in vitro o efeito do hiclato de doxiciclina na viabilidade de macrófagos, bem como o impacto desse antibiótico no reparo de feridas cutâneas em ratos.

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2 ARTIGO I

Does antibiotic use accelerate or retard cutaneous repair? A systematic review in animal models

Abstract

Background: The presence of infections is one of the main factors that leads to delays in healing or non-closure of cutaneous wounds. Although the goal of antibiotic use is to treat or prevent infection, there is currently no agreement on the effectiveness of these products. **Aim:** The aim of this study was to evaluate the efficacy of antibiotic use during the healing process of skin wounds in animal models not intentionally infected, as well as to analyze the advances and limitations of the studies carried out in this field. **Main methods:** This systematic review was performed according to the PRISMA guidelines, using a structured search on the MedLine (PubMed) and Scopus platforms to retrieve studies published until August 29, 2018, 13:35p.m. The studies included were limited to those that used excision or incision wound models and that were not intentionally infected. The data for the animal models, antibiotic used, and the main results of the studies were extracted, and compared where possible. Bias analysis and methodological quality assessments were examined through the SYRCLE's Risk of Bias tool. **Key findings:** Twenty-seven studies were selected. Overall, the effects of the antibiotic on the wound decreased inflammatory cell infiltration and promoted an increased number of fibroblasts, extracellular matrix constituents, re-epithelialization and tissue strength. A great deal of important information about the methodology was not presented, such as: the statistical analysis used, the animal model (sex and age), antibiotic dosage, blinding and randomization of the animals chosen. **Significance:** Based on the results found, we believe that antibiotic therapy can be considered a viable alternative for the treatment of cutaneous wounds. However, current evidence obtained from the methodological quality analysis points towards a high risk of bias. This is due to the incomplete characterization of the experimental design and treatment protocol, which compromises the reproducibility of the studies.

Keywords: wound healing, skin, cutaneous wounds, in vivo, antibiotic therapy.

Introduction

Skin plays an important role in protecting the body against aggressive agents, forming a barrier that prevents the entry and proliferation of pathogens [1]. When a tissue injury occurs, due to the action of microorganisms or trauma, the body initiates a series of complex events, aiming to reestablish the structure of the damaged skin tissue. The repair process can be divided into four stages: hemostasis, inflammation, proliferation and remodeling [2]. During hemostasis, platelets aggregate at the site of the lesion and initiate the process of blood coagulation, which also promotes vascular hemostasis and releases chemotactic factors that stimulate the migration and activation of immune cells that will be important for the next phase of the process [3]. In the next phase, leukocyte infiltration into the injured site occurs and these cells release proinflammatory cytokines, such as interleukins (IL1, IL6), tumor necrosis factor-α (TNF-α), and interferon gamma (IFN-γ); In addition, antimicrobial substances are also released, such as reactive oxygen species and proteases, that clean the wound and prepare the tissue for deposition of the extracellular matrix. In addition to inflammatory cells, fibroblasts are also attracted to the lesion site at this stage, which are mainly responsible for the synthesis of collagen and elastin [4,5]. The next phase is known as proliferative, characterized by the formation of granulation tissue, rich in blood vessels and collagen type III fibers [6]. This tissue is fragile and poorly resistant to traction, but it is the necessary basis for definitive tissue deposition. In addition, epithelium regenerates at this stage [7]. Remodeling is the final phase, where the fragile tissue is replaced by a strong tissue rich in collagen type I with a large number of cross-links forming bundles of fibers that give the new tissue mechanical strength [4,8].

The coordination and regulation of cellular, humoral and molecular processes can lead to perfect tissue regeneration [9]. However, factors such as the presence of infections, advanced patient age and metabolic disorders, can cause an imbalance in the repair process preventing adequate progression and wound closure [9,10]. Currently, a high prevalence of cutaneous lesions has been observed in elderly and diabetic populations, with a significant increase in the incidence of chronic wounds worldwide [10,11]. Often disguised as a comorbidity, chronic wounds represent a silent epidemic that worsens the patient's quality of life [12] and causes a significant financial burden on the public health system, since treatment for chronic wounds is expensive and time-consuming [13]. For example, in the United States of America, the Medicare dataset on all wound categories, including acute and chronic, estimated expenditures ranging from $28.1 to $96.8 billion on wound treatment [14]. Another report from Wales estimated a prevalence of 6% of chronic wounds at a cost of 5.5% for National Health Service

(NHS) [15]. Therefore, the economic impact generated by wounds is a concern. In addition to the costs involved in treating and managing the wounds, when not treated effectively, cutaneous lesions do not close and can progress to severe sepsis, amputation and even lead to patient mortality [16].

The progression of a wound to an infected state and consequent chronification of the lesion is determined by the ability of the host to generate an effective immune response, in addition to the amount of pathogens that come into contact with the injured tissue [17]. The exposed subcutaneous tissue provides a moist, warm and nutritious environment that is favorable to the proliferation of a wide variety of microorganisms [18]. To avoid colonization by pathogens and consequent chronification of lesions, there is a broad spectrum of therapies available on the market, but their effects on the repair process are still unclear. In this context, antibiotics have been growing as a therapeutic alternative. Some studies support the routine use of antibiotics in wound management due to their favoring cellular and vascular proliferation, thereby accelerating the closure of cutaneous lesions [19,20], as well as being effective in reducing infection [18]. Therefore, knowing that one of the major complications of wound healing is infection, the correct use of antibiotics can speed up wound healing and significantly reduce health care costs [21]. However, there is still a discussion regarding the effectiveness of using antibiotics in the treatment of not intentionally infected wounds due to their possible cytotoxic effect on fibroblasts and keratinocytes [22,23]. Given the uncertainties and controversies surrounding the role of antibiotics in treating cutaneous wounds, this review aimed to analyze the evidence regarding the use of antibiotics in the repair of cutaneous wounds by experimental incision or excision, in not intentionally infected animal models. Furthermore, the study evaluated the role of antibiotics in wound management and the relevance of the treatment. Methodological quality was also analyzed, together with the advances and limitations of these studies, identifying the main sources of bias.

Materials and methods

Focus question

The main question to be answered in this systematic review was: Does the use of antibiotics accelerate or slow cutaneous repair in animal models? Second, what are the main methodological parameters used to evaluate the evolution of the repair process in not intentionally infected animal models?

Search strategy

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [24] (Fig 1). The studies were selected through an advanced search on the platforms PubMed and Scopus, on August 29, 2018 13:35 p.m. Based on two search parameters, we devised a comprehensive search strategy for the retrieval of all relevant studies: (i) direct searches in electronic databases, and (ii) indirect screening of reference lists from all studies identified in the direct searches. For all databases, the search filters were based on four complementary levels: (i) animals, (ii) wound healing, (iii) skin and (iv) antibiotics. Search filters were initially developed for PubMed, the search algorithms [MeSH Terms] and [TIAB] were applied, to identify indexed records and those recently published in indexing processes, respectively. To detect all in vivo animal model studies in PubMed, a standardized and optimized animal filter was obtained [25]. The terms used to search on PubMed were adapted for the selection of Scopus publications, and the "animal model" filter is provided by the site itself. No date limit was applied. Languages were restricted to articles in English, Portuguese and Spanish. The abstracts of all articles chosen were interpreted to identify potentially eligible articles.

Two reviewers (LSA and RSA) conducted the literature search, removed duplicate 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 (LSA and RSA). The kappa test was done for the selection and data extraction (kappa = 0.839). Selections were then compared, and inconsistencies were resolved in consultation with three other reviewers (MMS, RDN and RVG).

Inclusion and exclusion criteria

Only original studies investigating the use of antibiotics in the cutaneous repair process of non-sutured incisional and excisional animal wound models were included. To identify the antibiotics, the List of Critically Important Antimicrobials for Human Medicine (WHO CIA List) guide developed by World Health Organization (WHO), the fifth revision of the WHO CIA List, published by WHO in 2016 was used [26]. We excluded from the review studies that used antimicrobial drugs not described by the aforementioned guide, studies that used only in vitro or ex vivo experimental models, studies with no full-text available, secondary studies (literature reviews, letters to the editor, case studies, comments and editorials), studies with plant species or peptides, studies with other organs or tissues, studies in diabetic animals and

wounds resulting from burns. Two reviewers (LSA and RSA) manually searched reference lists of studies selected in the previous step independently to find additional relevant articles.

Data extraction

Three independent reviewers (LSA, RSA and MMS) extracted the essential data grouped into four descriptive levels as follows: (i) publication characteristics (authors, date of publication, and country); (ii) characteristics of the animal models (species, sex, age, and weight); (iii) experimental interventions (asepsis, biopsy day, dermal wound instrument, wound size, number of wounds per animal, anesthesia); (iv) information about antibiotic treatment (name of the antibiotic used, dose, frequency of administration, route of administration, pharmaceutical form and treatment in the control group); (v) the results from groups treated with antibiotics for all the studies were pooled (synthesis of extracellular matrix components, neovascularization, wound strength, time to wound closure). Any inconsistencies regarding the extracted data were resolved during discussions with two additional reviewers (RVG and RDN).

Assessment of risk of bias in included studies

To assess the risk of bias in the studies included, SYRCLE's Risk of Bias (RoB) tool, designed specifically for animal studies, was used [25]. The following methodological domains based on RoB were evaluated. Consider selection bias: "Was the allocation sequence adequately generated and applied?", "Were the groups similar at baseline or were they adjusted for confounders in the analysis?", "Was the allocation to the different groups adequately concealed?"; Consider performance bias: "Were the animals randomly housed during the experiment?", "Were the caregivers and/or researchers blinded regarding which intervention each animal received during the experiment?"; Consider detection bias: "Were animals selected at random for outcome assessment?", "Was the outcome assessor blinded?"; Considers attrition bias: "Were incomplete outcome data adequately addressed?"; Considers reporting bias: "Are reports of the study free of selective outcome reporting?"; Considers other biases: "Was the study apparently free of other problems that could result in high risk of bias?"; The overall study quality indicators: "Was randomization at any level the experiment indicated?" and "Was it stated that the experiment was blinded at any level?". The items in the RoB tool were scored with "yes" (low risk of bias); "no" (high risk of bias); or "unclear" (indicating that the item was not reported, and therefore, the risk of bias was unknown).

Results

Characteristics of publications

The initial research resulted in 1536 studies, with 934 from PubMed and 602 from Scopus. Out of these, 263 were excluded because they were duplicate studies. After reading the titles and abstracts, 47 relevant studies were selected and read in full, and their references checked. Finally, 27 studies fully met the inclusion criteria and were included in the systematic review. The process of selecting articles is shown in a flowchart (Fig 1). The filters applied in each database and the flowchart indicating the search structure are shown in supplementary S1 Table.

The 27 studies were conducted in eight different countries: the United States of America (40.47%, n= 11), South Korea, India (14.81%, n = 4 each), Taiwan (11.11%, n = 3), China (7.41%, n = 2), Turkey, South Africa and Australia (3.70%, n = 1 each).

An absence of an animal ethics committee was observed in 25.93% of studies (n=7) and statistical analysis of the individual studies was performed in 77.78% of studies (n = 21), while 22.22% of the studies (n = 6) did not report any statistical comparison of the data (S2 Table).

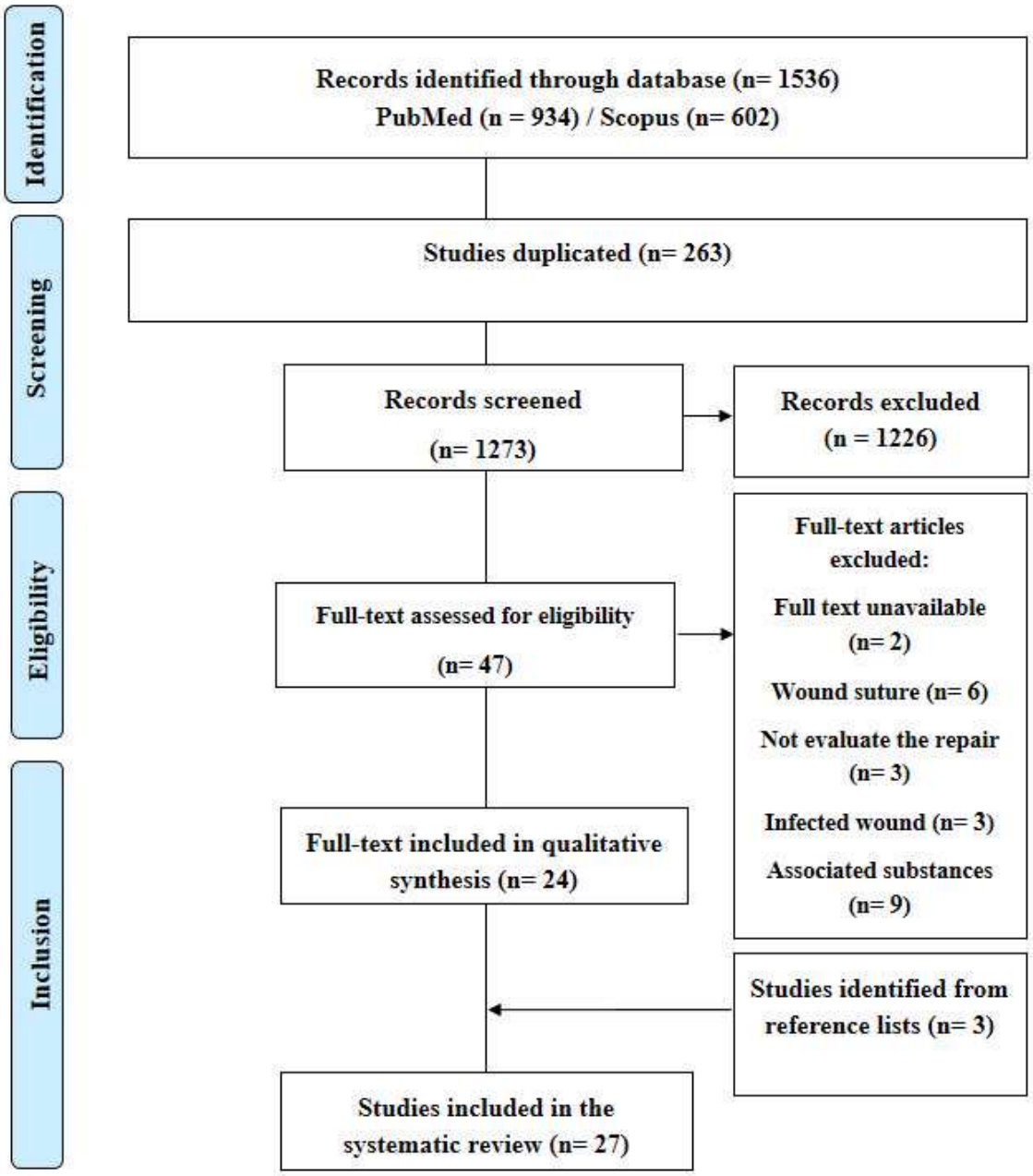

Fig 1. PRISMA diagram. Different phases of selection of studies for conducting qualitative and quantitative analyses. Flow diagram of the systematic review literature search results. Based on 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement'. http://www.prisma-statement.org. From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009).

Characteristics of experimental animals

As shown in Supplementary S2 Table, rats were the main animal model used (55.56%, n = 15), followed by pigs (18.52%, n = 5), mice (11.11%, n = 3), horses and rabbits (5.56%, n = 2 each). The proportion of sex of animals was 44.44% male (n = 12), 22.22% female (n = 6) and 3.70% both (n = 1). Eight studies did not specify the sex of the animals (29.63%). The age of the animals was only specified in 22.22% of the studies (n = 6). In 14.81% of studies (n = 4)

adult or young animals were described without a specific age while 62.96% did not report this information. Body weight data was specified in most studies (85.19%, n = 23) while 14.81% of studies omitted this information (n = 4). 25.93% of studies omitted the total number of animals in the experiment (n = 7). In the studies where this information was reported, 10% used up to 5 animals (n = 2), 40% ranged from 6 to 15 animals (n = 8), 30% ranged from 16 to 25 animals (n = 6) and 20% used more than 25 animals (n = 4) (S2 Table).

Wound characteristics

59.26% of the studies (n = 16) reported the type of asepsis performed prior to wounding, with ethanol being the substance most commonly used (43.75%, n = 7), followed by povidone-iodine (PI) (18.75%, n = 3). Combinations such as saline, PI + ethanol, PI + saline, chlorhexidine gluconate (CHX), CHX + saline and CHX + saline + ethanol were used in 4.55% of studies (n =1 each). The day of the experiment when the biopsy was performed was specified in 74.07% of studies (n = 20), whereas 18.52% did not biopsy the wound (n = 5) and 7.41% did not indicate the day of the procedure (n = 2). The instrument used in wounding was reported in 51.85% (n=14) of studies. 35.71% of studies (n = 5) used scalpels, 21.43% used punch biopsy (n = 3), 14.29% used scissors (n = 2), 14.29% used dermatome (n = 2) and 7.14% used punch biopsy + scalpel or dermatome + scissor (n = 1 each). All studies reported the wound size while the number of wounds made was reported in 85.19% (n = 23). 25% of studies made two wounds on the same animal (n = 6), 20.83% one or four wounds (n = 5 each), 8.33% six wounds (n = 2) and 4.17% made 12, 16, 20, 36, 45 or 150 wounds per animal (n = 1 each). The anesthetic specifications were reported in 96.30% (n = 26) of the studies with the most commonly cited being: ketamine + xylazine and pentobarbital (11.54%, n = 3 each) (S3 Table).

Antibiotic characteristics

Related information in studies

Fifteen types of antibiotic were observed in the selected studies, with the most frequently used being silver sulfadiazine (28.2%, n = 11), followed by gentamicin (12.8%, n = 5), ciprofloxacin, mupirocin and bacitracin (7.7%, n = 3 each). The antibiotics clindamycin, neomycin, nitrofurazon and polymycin b were used in 2 studies each (5.1%). The antibiotics amikacin, doxycycline, fusidic acid, moxifloxacin, rifamicin, and vancomycin were only used in one study each (2.6% each) (Fig 2 a).

74.07% (n=20) of studies reported the concentration of antibiotic used while 25.93% (n=7) of studies did not indicate this information. The frequency of antibiotic administration was reported in 25 studies (92.59%). The most frequent administration timeframe was a single day (48%, n = 12), 28% ranged between two to seven days (n = 7), 16% between eight to fourteen days (n = 4) and 8% longer than 15 days (n = 2). The most common administration route was topical (88.89%, n = 24), followed by oral (7.41%, n = 2) and intravenous (3.7%, n = 1). The main mode of administration of the antibiotic was through curative (48.15%, n=13) on the injured skin, while 25.93% (n=7) administered the antibiotic in a cream, 14.81% (n= 4) in an ointment, 7.41% administered it in powdered form dissolved in the drinking water of the animals and 3.7% (n=1) administered it in liquid form, by means of injection.

Most studies used more than one control group and the interventions performed in these groups were: untreated (30.95%, n = 13), vehicle (28.57%, n = 12), gauze (16.67%, n 7), commercial product (9.54%, n = 4), saline (4.76%, n = 2), Ky lubricant®, bandaged, Vaseline gauze, hydrocolloid, povidone-iodine (PI) (2.38%, n = 1 each) (Table 1).

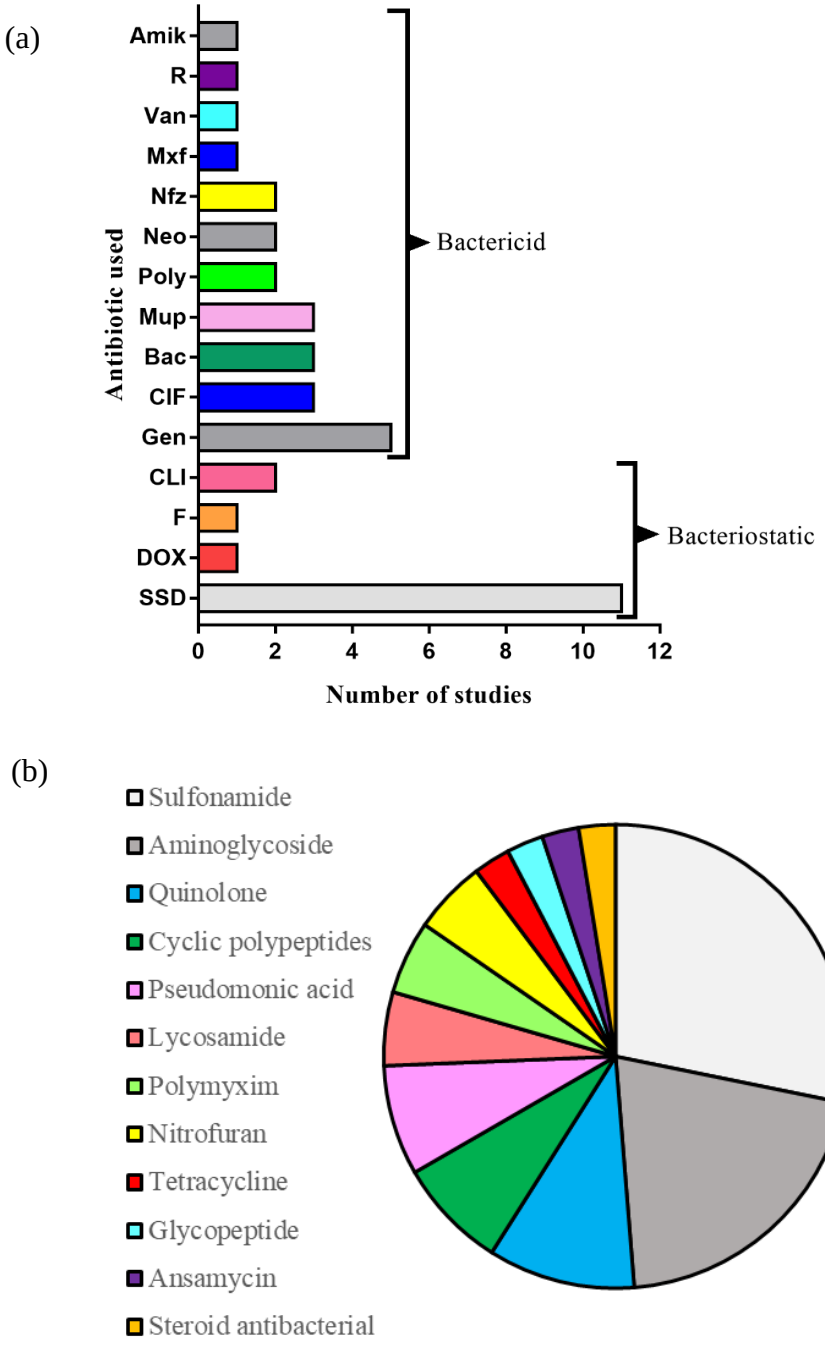

Fig 2. Characteristic of the antibiotic used in the studies of this systematic review that evaluated the effect of the antibiotic on the healing of not intentionally infected wounds. (a) antibiotics used in the studies, (b) classes of antibiotics. Van = vancomycin, Amik = Amikacin, Gen = gentamicin, Mxf = Moxifloxacin, Nfz = Nitrofurazone, SSD = silver sulfadiazine, SA = sodium alginate, CLI = clindamycin, CIF = ciprofloxacin, Dex = dexamethasone, Mup = mupirocin, Bac = bacitracin, Poly = polymyxin B, R = rifamycin, F = fusidic acid, Neo = neomycin, DOX = doxycycline.

Table 1. Description of the main characteristics of the antibiotic treatments used in the studies of this systematic review that evaluated the effect of the antibiotic on the healing of not intentionally infected wounds.

Antibiotic						
Animal model: Rat						
Reference	**Antibiotic**	**Classe**	**Concentration**	**Frequency (d)**	**Format**	**Control**
Leitch, et al. 1993 [27]	Silver sulfadiazine (SSD)	Sulfonamide	?	?	Cream	Untreated
Heggers et al. 1995 [28]	Silver sulfadiazine (SSD)/ mupirocin (Mup)/ clindamycin (CLI)	Sulfonamide/ pseudomonic acids/ lincosamide	1- 2%	14	Cream/ ointment	Untreated
Choi et al. 1999 [29]	Silver sulfadiazine (SSD)	Sulfonamide	0.4 mg/cm^2	1	Curative	Vaseline gauze
Muller et al. 2003 [30]	Silver sulfadiazine (SSD)	Sulfonamide	0.5/ 1%	14	Cream	Sal/ vehicle
Kim, et al. 2008 [31]	Clindamycin (CLI)	Lincosamide	?	1	Curative	Gauze
Kim, et al. 2008 [32]	Nitrofurazone (nfz)	Nitrofuran	?	1	Curative	Vehicle
Simpson, et al. 2008 [33]	Bacitracin (Bac)+ neomycin (Neo)+ polymyxin B (Poly)	Cyclic polypeptide/ aminoglycoside/ polymyxin	?	9	Ointment	Untreated
Hwang et al. 2010 [34]	Gentamicin (gen)	Aminoglycoside	0.1%	1	Curative	Gauze/ commercial product/ vehicle
Lin et al. 2010 [35]	Gentamicin (gen)	Aminoglycoside	0,50 mg/ mL	1	Curative	Gauze
Huang et al. 2012 [36]	Gentamicin (gen)	Aminoglycosides	0.05%	1	Curative	Gauze/ hydrocolloid dressing
Gurel et al. 2013 [37]	Rifamycin (R)/ fusidic acid (F)	Ansamycin/ steroid antibacterial	0.1 cm^3/ 0.25 g	7	?	Sal
Mittal and Kumar 2014 [38]	Gentamicin (gen)	Aminoglycoside	590 µg/ mg	1	Curative	Untreated
Princely et al. 2015 [39]	Gentamicin (Gen)	Aminoglycoside	?	1	Curative	Vehicle/ PI
Fu et al. 2016 [40]	Moxifloxacin (Mxf)	Quinolone	2%	1	Curative	Commercial product/ vehicle/ untreated
Li et al. 2017 [41]	Ciprofloxacin (CIF)	Quinolone	0,9%	7	Curative	Gauze
Animal model: Pig						
Reference	**Antibiotic**	**Classe**	**Concentration**	**Frequency (d)**	**Format**	**Control**
Geronemus, et al. 1979 [42]	Bacitracin (Bac)+ neomycin (Neo)+ polymyxin B (Poly)/ nitrofurazone (Nfz) / silver sulfadiazine (SSD)	Cyclic polypeptides/ aminoglycoside/ polymyxin/ nitrofuran / sulfonamide	?	6	Ointment	Untreated/ vehicle

Watcher and Wheeland 1989 [43]	Bacitracin (Bac)/ silver sulfadiazine (SSD)/ mupirocin (Mup)	Cyclic polypeptides/ sulfonamide/ pseudomonic acid	1- 2%	27	ointment/ cream	K-Y lubricant/ untreated
Singer, et al. 1999 [44]	Silver sulfadiazine (SSD)	Sulfonamide	?	4	?	Gauze
Faucher, et al. 2010 [45]	Silver sulfadiazine (SSD)	Sulfonamide	1%	1	Curative	Gauze/ vehicle/ untreated
Theunissen et al. 2016 [46]	Mupirocin (Mup)/ silver sulfadiazine (SSD)	Pseudomonic acid/ sulfonamide	1- 2%	28	Cream	Untreated
Animal model: Mice						
Reference	**Antibiotic**	**Classe**	**Concentration**	**Frequency (d)**	**Format**	**Control**
Hebda et al. 2003 [47]	Doxycycline (DOX)	Tetracycline	2 mg/ml	3	Powder	Vehicle
Zhang et al. 2015 [48]	Vancomycin (Van)	Glycopeptides	4 mg/ml	12	Powder	Vehicle
Tummalapalli et al. 2016 [49]	Ciprofloxacin (CIF)	Quinolone	0.5-2.5%	1	Curative	Commercial product/ vehicle/ untreated
Animal model: Rabbit						
Reference	**Antibiotic**	**Classe**	**Concentration**	**Frequency (d)**	**Format**	**Control**
Kataria et al. 2014 [50]	Ciprofloxacin (CIF)	Quinolone	32–35 mg/ mL	1	Curative	Commercial product/ vehicle/ untreated
Qian, et al. 2017 [51]	Silver sulfadiazine (SSD)	Sulfonamide	0.01- 1%	2/7	Cream	Vehicle
Animal model: Horse						
Reference	**Antibiotic**	**Classe**	**Concentration**	**Frequency (d)**	**Format**	**Control**
Berry and Sullins 2003 [52]	Silver sulfadiazine (SSD)	Sulfonamide	1%	?	Cream	Bandaged/ untreated
Edwards-Milewski et al. 2016 [53]	Amikacin (amik)	Aminoglycoside	5 mg/ kg	3	Liquid	Untreated

PI= povidone-iodine, Sal = saline.

Antibiotic identification

The antibiotics were divided into 12 antibiotic classes (Fig 2 b). Most antibiotics present bactericidal effects (66.7%, n = 8), while others present bacteriostatic action (33.3%, n = 4) (Fig 2 a).

The action mechanism for most antibiotics is inhibition of bacterial protein synthesis (38.5%, n = 15), followed by inhibition of folic acid metabolism required for the synthesis of bacterial DNA and RNA (28.2%, n = 11), by action on bacterial nucleic acids, either by inhibiting their synthesis or causing damage (17.9%, n = 7), by inhibiting bacterial cell wall synthesis (10.3%, n = 4) or by alteration of the bacterial cell cytoplasmic membrane (5.1%, n = 2).

The class of antibiotic most commonly used in the studies was sulfonamide (28.2%, n = 11), which was represented only by silver sulfadiazine. Results in which healing was favored (n = 3) showed an increase in the rate of re-epithelialization, number of fibroblasts, matrix components and reduction of wound area. Some studies also showed no effect on healing time for this antibiotic (n = 6) and that it can lead to repair delays (n = 2), with a reduction in rates of re-epithelialization and an increase of wound half-life and rupture strength.

The second most used class in the studies was the class of aminoglycosides (20.5%, n = 8), which was represented by gentamicin, amikacin and neomycin. In studies where this class led to increased healing rates (n = 5), there was increased collagen production, re-epithelization, and proliferation of fibroblasts and blood vessels. In addition, there was a reduction of inflammation and wound area. In studies where the healing time did not differ from the control (n = 1), the production of extracellular matrix and the area and contraction of the wound did not change, however there was an increase in inflammatory cells. There was no delay in healing in any study. An association of the aminoglycoside, cyclic polypeptides and polymyxim classes was observed in two studies. The association of these three antibiotics led to improved healing, with increased re-epithelialization (n = 1). In another study the healing time was unaffected (n = 1), showing the same rates of re-epithelialization and wound contraction as the control group.

The third most commonly used class of antibiotics was the quinolone class (10.3%, n = 4), and was represented by ciprofloxacin and moxifloxacin. When healing was favored (n = 3), the area of the wound and inflammation were reduced, as well as presenting an increase in the extracellular matrix. In the study in which the healing time was not affected (n = 1), there was an increase in collagen synthesis and organization, increased vascularization and number of fibroblasts. No healing delay was observed in any study.

The class of pseudomonic acid, represented only by mupirocin, and the cyclic polypeptides class, represented only by bacitracin, were analyzed in three studies each (7.7%). In the pseudomonic acid class, when the healing time was reduced (n = 1) there was an increase in granulation tissue, fibroblasts, extracellular matrix formation and re-epithelialization rate. In the study where the healing time did not differ from the control group (n = 1), the re-epithelialization rate was unaffected, but there were delays in wound contraction. When this class of antibiotics presented delays in healing time (n = 1), the rupture force of the wound increased. The cyclic polypeptide class was used in only one study without being associated with other antibiotics and did not alter the healing time in relation to the control. In addition, a smaller wound contraction was observed, even though greater re-epithelialization was also noted.

The antibiotic classes lycosamide, represented only by clindamycin, and nitrofuran, represented only by nitrofurazone, were analyzed in two studies each (5.1%). In the study in which lycosamide led to decreased healing time (n = 1), there was a reduction of inflammation and wound area and increased granulation tissue. In the study where a delay in healing time was observed (n = 1), lyncosamide increased the rupture force of the wound. In the study that applied Nitrofuran, a reduction in healing time (n = 1) was observed, with a consequent reduction of wound area and inflammatory infiltrate. However, it was also seen that this class led to a decrease in re-epithelialization and consequent delays in healing (n = 1).

The classes tetracycline, glycopeptide, ansamycin and antibacterial steroid were used in one study each (2.6%). The tetracycline class was represented by doxycycline and did not show changes in healing time, but presented increased collagen organization and consequently, increased rupture force of the wound force rupture. The glycopeptide class was represented by vancomycin, leading to delayed healing time and decreased expression of RegIIIγ, the secreted C-type lectin. The class ansamycin represented by rifamycin led to lower re-epithelialization, inflammatory infiltrate, vascularization and fibroblast numbers and as a result, healing was faster than in the control group. The antibacterial steroid class was represented by fusidic acid with a delay in healing time being observed due to the greater intensity of fibroblast accumulation, which caused a longer proliferative phase, with less vascularization and inflammation.

Main outcomes

In most of the studies, the process of cutaneous repair was accelerated by antibiotic treatment. Even in the studies in which the antibiotics did not reduce healing time in relation to

the control groups, antibiotic treatments generally led to positive outcomes, increasing extracellular matrix components and the rupture force of the wound. In the antibiotic formulations found in the studies, we observed that 45.71% (n=16) of antibiotics tested presented a reduction in healing time, 34.29% (n=12) did not alter the healing time and, 20.0% (n=7) led to a slower healing time.

In studies where healing time was shortened, an increase in fibroblasts, extracellular matrix components and re-epithelization were observed, as well as reductions in inflammatory infiltrate and the wound area. In the studies where healing time was unaffected by the administration of antibiotics, the treatment favored collagen organization and consequently an increase in wound force, neovascularization and an increase in fibroblasts. The results described above are shown in Fig 3. Considering the articles that described the antibiotics, and which presented a longer healing time, the main results that explain this finding were an increase in wound half-life, wound area and a reduction in re-epithelialization. Additionally, there was an increase in tissue resistance, because the rupture force of the wound increased (Table 2).

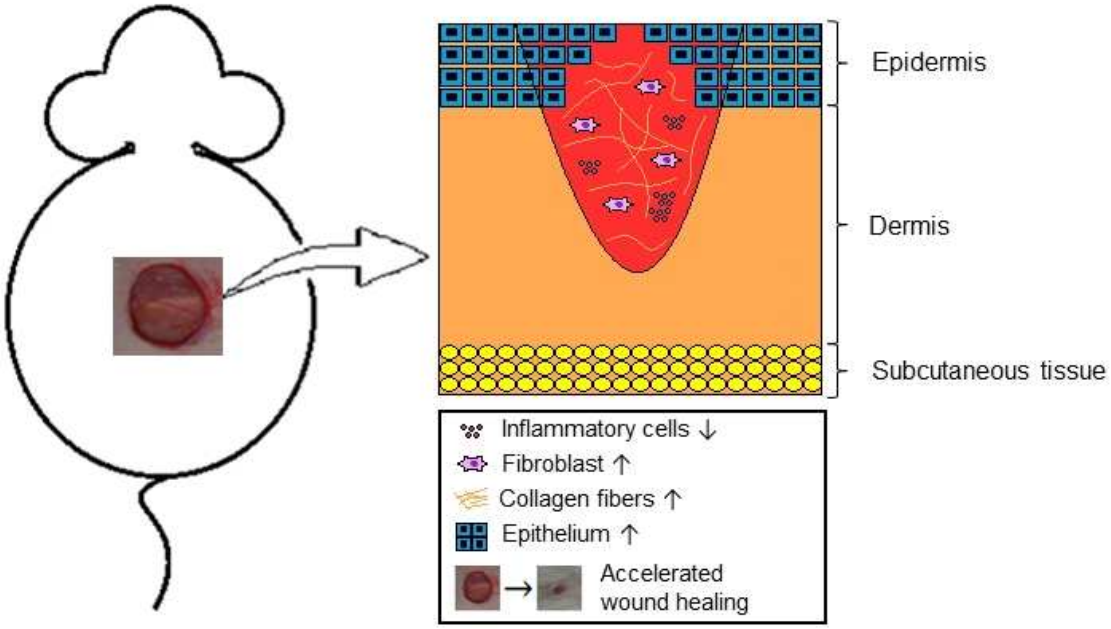

Fig 3. A schematic diagram of the general action of antibiotics in healing of not intentionally infected cutaneous wounds in animal models.

Table 2. Main results of the action of the classes of antibiotics on wound healing in animal models not infected. The results were separated according to healing time in relation to the control.

Class	Healing time	Measure outcomes
Sulfonamide	Reduction (n=3)	↑ Reepithelialization, fibroblasts, ECM ↓ Wound area
	Similar (n=6)	= Reepithelialization, wound area, wound contraction
	Increase (n=2)	↑ Wound half-life, rupture strength ↓ Reepithelialization
Aminoglycoside	Reduction (n=5)	↑ Fibroblasts, reepithelialization, ECM, blood vessels ↓ Wound area, inflammatory cells
	Similar (n=1)	= Wound area, Wound contraction ↑ inflammatory cells
Quinolone	Reduction (n=3)	↑ ECM ↓ Wound area, inflammatory cells
	Similar (n=1)	↑ ECM, blood vessels, fibroblasts
Pseudomonic acid	Reduction (n=1)	↑ Granulation tissue, fibroblasts, ECM, reepithelialization
	Similar (n=1)	= Reepithelialization ↓ Wound contraction
	Increase (n=1)	↑ Wound area, rupture strength, wound half-life
Lycosamide	Reduction (n=1)	↑ Granulation tissue ↓ Wound area, inflammatory cells
	Increase (n=1)	↑ Wound area, rupture strength, wound half-life
Nitrofuran	Reduction (n=1)	↓ Wound area, inflammatory cells
	Increase (n=1)	↓ Reepithelialization
Tetracycline	Similar (n=1)	= Wound area, reepithelialization ↑ Rupture strength
Glycopeptide	Increase (n=1)	↑ Wound area ↓ Expression of RegIIIγ
Ansamycin	Reduction (n=1)	↓ Reepithelialization, inflammatory cells, blood vessels, fibroblasts
Steroid antibacterial	Increase (n=1)	= ECM ↑ Fibroblasts ↓ Reepithelialization, inflammatory cells, blood vessels
Cyclic polypeptides	Similar (n=1)	↑ Reepithelialization ↓ Wound contraction
Cyclic polypeptides + Aminoglycoside + Polymyxim teroid antibacterial	Reduction (n=1)	↑ Reepithelialization
	Similar (n=1)	= Reepithelialization, wound area, wound contraction

ECM= extracellular matrix

Risk of Bias and Methodological Quality Assessments

The detailed results for the analysis of bias are shown in the Fig 4. No studies fulfilled all the methodological criteria analyzed. In relation to selection bias, the sequence generation process was not reported in 77.78% studies (n=21). In terms of the animal's characteristics, that is, their similarity to each other (Q2), 22 studies (81.48%) did not report this information clearly. 21 studies (77.78%) did not report information regarding the allocation concealment (Q3). None of the articles reported random housing or blinding of caregivers (Q4 and Q5, respectively) and as such, the outcome was evaluated as presenting high risk of bias. 24 studies (88.89%) did not report random outcome assessment for detection bias, for relevant outcome measures (Q6). In addition, the outcome assessor was not reported to have been blinded in 21 studies (77.78%; Q7). 15 studies (55.56%; Q8) showed incomplete outcome data. 6 studies (22.22%) presented a high risk for reporting bias (Q9). In addition, 16 studies (59.26%; Q10) presented other potential sources of bias. Two quality indicators were used to assess the methodological quality of the studies. 51.85% of the studies (n=14) reported no randomization at any level of the experiment (Q11). 74.07% of studies (n= 20; Q12) did not report blinding. The analysis of the individual studies found no relation between risk of bias and the year the studies were published (Fig 5). Thus, the most recent studies do not describe the methodological quality criteria, in comparison with older studies.

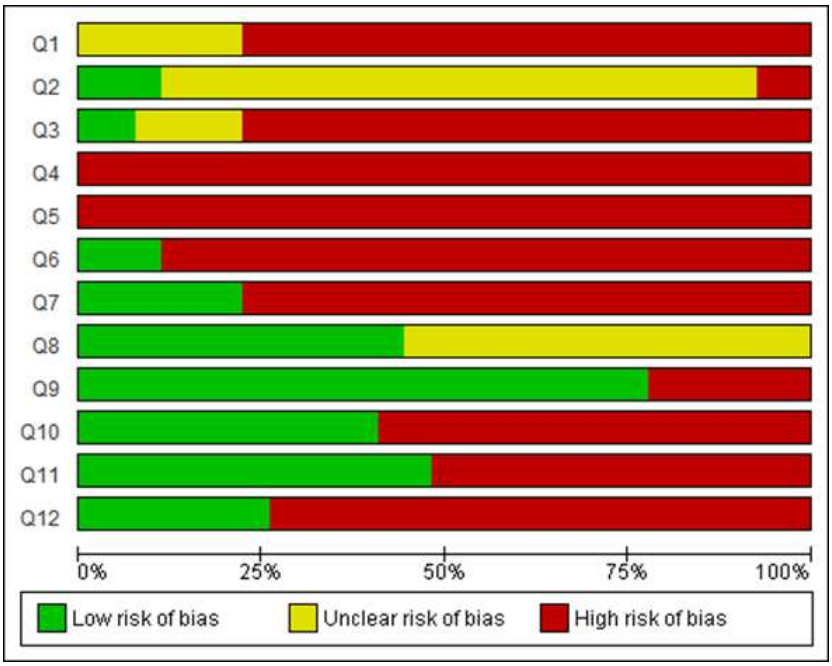

Fig 4. Results for the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the effect of the antibiotic on the healing of not intentionally infected wounds. The items in the Systematic Review Centre for Laboratory Animal Experimentation (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 that the item was not reported, resulting in an unknown 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. The overall study quality indicators (Q11–Q12) were scored with "yes" when reported or "no" when not reported. 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 to the different groups adequately concealed?; Q4: Were the animals randomly housed during the experiment?; Q5: Were the caregivers and/or investigators blinded from knowledge regarding 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?; Q10: Was the study apparently free of other problems that could result in high risk of bias?; Q11: Was it stated whether the experiment was randomized at any level?; Q12: Was it stated whether the experiment was blinded at any level?

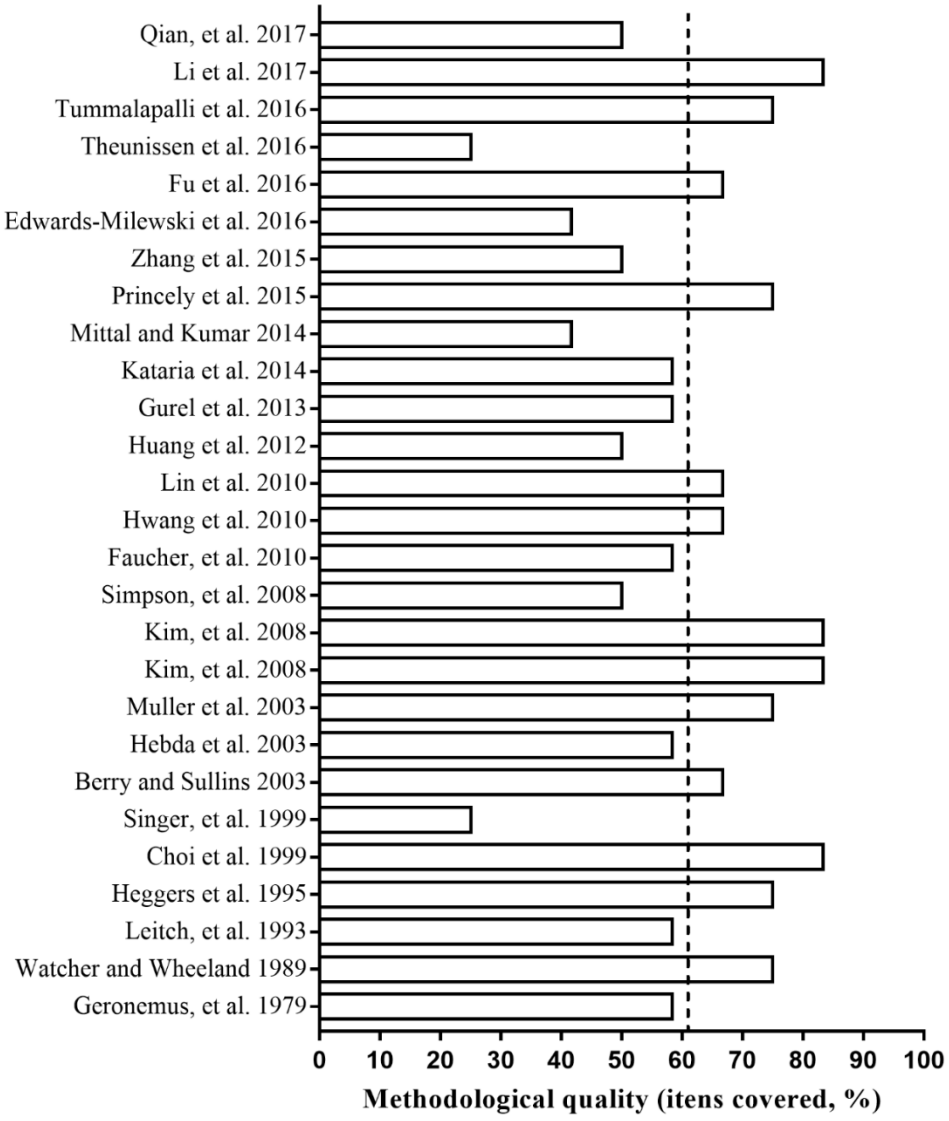

Fig 5. Analysis of high risk of bias of each study included in the review: year of publication versus high risk of bias. Based in the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) Risk of Bias.

Discussion

In our study, we conducted a systematic revision to investigate antibiotic use in the healing of not intentionally infected cutaneous wounds. Our results showed that antibiotic use can accelerate the process of cutaneous healing, reducing the wound area and inflammatory infiltration, as well as increasing the number of fibroblasts, the synthesis of extracellular matrix components (MEC), the formation of epithelial tissue and the force of wound closure. However, a small number of studies showed that antibiotic therapies can also negatively affect healing, leading to slower wound closure. These findings, given that the inadequate use of antibiotics can lead to an increase in bacterial resistance [54], mean that the choice of antibiotic therapy for treating cutaneous wounds should be given careful consideration. Currently, the global consumption of antibiotics is increasing considerably. Between 2000 and 2015, there was a 65% increase in the use of these compounds [55], with the USA standing out as the biggest consumer of antibiotics in the world, when taking into consideration other high income countries [55]. This finding was confirmed by our research, as the greater part of the studies observed in this revision were carried out in the United States, followed by South Korea and India.

During wound healing, there are some steps that are extremely important for the formation of a strong scar free of infection. In this context, we can highlight the inflammatory phase, which is characterized by the recruitment of the innate immune system, which acts against attacks by invasive pathogens, helping to remove dead tissue [56, 57]. However, prolonged inflammation is prejudicial and can hamper the progress of the healing process [4]. Our findings showed that antibiotic use promotes a reduction in the number of inflammatory cells and, therefore, probably favors the transition from the inflammatory to the proliferative phase. In addition, we also observed greater tissue re-epithelialization following the use of antibiotics, which favors cellular proliferation, mainly of fibroblasts and consequently the synthesis of extracellular matrix components. Another important finding was the increase in tissue force following the administration of antibiotics, possibly due to the greater percentage and organization of collagen synthesized by the fibroblasts when compared to the control groups. Therefore, in general, antibiotics are an interesting alternative for wound treatment, given that they favor the transition from the inflammatory to the proliferative phase, the closure of the wound surface, the formation of granulation tissue and increased resistance of the scar.

In recent years, advances in understanding through research related to wound healing has led to the development of different therapies, in search of lower costs together with patient

wellbeing [58,59]. Traditional treatments, such as bandages, cotton wool and gauzes only provide initial mechanical protection, but certain characteristics restrict their use, such as their absorbent properties and permeability. This means the wound becomes an environment favorable to the growth of pathogenic microorganisms, in addition to adhering to the surface of the wound, which can induce epithelial trauma during removal [58]. In this context, the use of antibiotic therapies is common and necessary to slow the proliferation of pathogenic microorganisms that can invade the tissue and cause infections, leading to an excessive inflammatory response that could cause chronification of the healing process [59]. Our results showed that antibiotic use, mainly in a curative application based on hydrogel formulations, are important to speed up cutaneous healing. This type of therapy is growing, probably seeking to overcome problems related to traditional treatments, in addition to giving the wound a moist and hydrated surface, ideal for accelerating the process of cutaneous repair.

The findings of our study show that the materials used for the development of new cures for wounds were based on synthetic polymers, cellulose, and gelatin, amongst others. In the majority of the studies where a cure was developed in association with antibiotics, improvements in the tissue repair process were observed. However, to determine if the antibiotic contributed to the positive outcomes of the cure, an adequate control group should have been implemented. The control group needed to present the same elements as the experimental group, except for the evaluated compound [60], in this case, antibiotics. It is interesting to note that few studies reported the presence of a control group with these characteristics, suggesting a need to improve methodological standards in studies investigating the use of antibiotics in healing. The absence of this group is considered a serious methodological failing, since it leads to the obtainment of false-positive results, as well as hampering the determination of the beneficial effects of the substance of interest [61].

Our revision had as its object studies employing animal models, and one of the biggest advantages of the use of these models for wound healing was that they allow for histological monitoring of the wound healing process. Additionally, they allow for the realization of macroscopic, biochemical and biomechanical measurements [62]. In our research, the animal most commonly used in the studies was the rat, possibly due to the low cost, and easy handling and accessibility, allowing researchers to use a relatively large number of animals for their experiments, thereby generating a greater degree of reliability in the results [63]. In addition, the area of dorse is higher when compared to mice. Pig models were the second most used animal, possibly due to similarities between pig and human skin, mainly in terms of general structure, thickness, follicle content, capillaries, pigmentation, collagen content and lipid

composition [64]. Some studies used larger animals, however, these studies used a smaller number of animals per group, possibly as a result of the cost, due to its not being practical for the majority of research installations, as well as presenting difficulties with handling during the realization of procedures [65]. In this context, the reduction in the number of experimental animals may be a complicating factor in research due to increased risk of obtaining inconclusive results [66].

Considering wound models, we observed that despite studies that evaluated wounds by incision being included, the greater majority used the excision model when investigating the tissue repair process. The excisional wounds are valid and reproducible models, being very useful in the analysis of healing [67]. In addition to the wound model, the number of wounds per animal is also an important parameter when dealing with studies of healing. In our revision, we found that a considerable number of wounds were made on the same animal, and consequently, these wounds were not located on the same bodily region. The same location for biopsy should be maintained between the groups due to wound contraction, re-epithelialization rates, and total tissue repair varying depending on location [68]. Additionally, the realization of many wounds can increase stress on the animal, and independent of the duration of the stress factor, lead to alterations in local chemical mediators and of the cells involved in the initial stages of wound healing, thereby compromising the results [68].

The class of antibiotics most commonly used in the studies in this revision was sulfonamide, represented by silver sulfadiazine. The prevalence of this antibiotic amongst these studies can be explained by its extensive medical use, being the most widely used topical antimicrobial for burns in recent decades [69]. Topical antibacterial agents, specifically silver sulfadiazine cream, are used to reduce bacterial count. To the contrary of other means of application, the topical route allows the antibiotic to penetrate adequately into the open and granulous wound, being able to exercise a direct bacteriostatic or bactericidal effect on an ample spectrum of gram-positive and gram-negative organisms [70]. Therefore, topical use allows the antibiotic to reach high local concentrations of the drug with minimal systemic absorption, thereby minimizing the risk of adverse systemic effects. Oral administration was performed in two studies by dissolving the pharmacological agent in the animal's drinking water. This form of administration limits the analysis since it does not precisely determine the quantity of antibiotic ingested by the animal. Additionally, given that no calculation of water consumption was carried out, these studies cannot be reproduced, due to the average consumption of the medication being unknown. Furthermore, this lack of control represents a methodological failing, since it hampers the attribution of the results derived directly from antibiotic use.

Administration via the intravenous route, on the other hand, was reported for only one study. This route allows the antibiotic to be distributed throughout the organism, meaning that all wounds on the animal receive the medication, including the control wound when made on the same animal [53].

In the last years, advances in understanding regarding wound healing has led to the development of innumerable therapies, but the enormous costs associated with this disease and the seeks to promote patient well-being have driven new research efforts to find the ideal treatment. As a consequence, a variety of new preparations, curative materials and advanced methods of debridement have been presented in scientific articles through experiments involving animal models. Therefore, this revision brought together published studies that used animal models to investigate antibiotic use in the repair of incisional and excisional wound models. In terms of the methodological analyses performed for these studies, generally the time for wound closure, tissue resistance and histomorphology were evaluated (quantification of matrix components, neovascularization, cells from the lesion area). The results showed that antibiotics led to a reduction in wound area, and a consequent increase in proliferation of fibroblasts and of extracellular matrix components, and an increase in collagen organization, giving the wound greater resistance. Neovascularization was found to be variable between the studies, with molecular analysis being necessary to understand the action of these antibiotics on the regulation of angiogenesis. The methodology used to analyze tissue repair in the majority of the studies was considered poor due to not investigating the molecular or biochemical effects on healing, for example, the action of cytokines and oxidative stress, in addition to poorly explaining the mechanisms involved in wound healing.

The results found in this review showed that antibiotic therapy favors cutaneous healing processes by reducing inflammation and increasing cell and vessel proliferation. However, it is important to highlight that the preventive use of antibiotics should be considered with caution. In the age of multiple drug resistance for antibiotics, the preventive use of antibiotics for treatment of cutaneous lesions is not advisable. Nonetheless, we know that antibiotic therapy is indicated as an adjunct to wound care as it promotes infection control in open wounds, burns and trauma. In this context, antibiotic use has a beneficial effect on the wound healing process, but with the aim of assisting other drugs that are elective to accelerate closure and recover matrix components. Therefore, we believe that antibiotic therapy should be used to treat wounds when there is a risk of infection and not as an elective therapy for treatment of skin lesions. This is important to avoid the natural selection and multiplication of resistant bacteria [71]. Bacterial resistance increases treatment costs, prolongs hospital stays and may consequently

increase mortality rates [72]. Therefore, we believe that the rational use of antibiotics and the continued development of alternative treatments are necessary to decrease antibiotic resistance. Among the alternative therapies available today to maximize the effect of antibiotics, or even decrease their use, are immunity modulating agents, bacteriophages and their lysines, antimicrobial peptides, pro and pre-symbiotics, plant extracts, pathogenicity inhibitors (quorum detection bacterial, biofilm and virulence), food enzymes [73], phytochemicals and metals [74].

Guide to reporting relevant information

Based on the findings of this review, we have created a guide that includes a list of elements that should be reported, quantitatively, in order to make it easier to compare results between articles that test the effects of antibiotics on cutaneous wound healing (S4 Table)

Limitations

Systematic revisions are considered high level studies that allow for the individual evaluation of studies in a blind manner using specific tools [75]. Such characteristics lead to a more inclusive, non-tendentious approach, to provide the reader with a broad understanding of the studies included in systematic revisions. One of the limitations was that this systematic review has not been registered online, once the register it is an important initiative to promote the quality of scientific publication, promote transparency, reduce duplication, and minimize bias in reviews. The results presented in this study are important and valuable to manage antibiotic use in cutaneous wound treatment. However, they should also be treated with caution, since the majority of the studies presented significant methodological variability, mainly in terms of the control and association or not of other compounds, hampering comparison and categorical conclusions regarding the benefits of antibiotic interventions.

The differences between the days for the realization of the biopsy, the use of different animals and the highly variable wound sizes between studies, meant that the days for wound closure were not comparable between the studies included in this revision. An important observation was that a large number of the studies did not demonstrate the realization of a statistical analysis of their data, reducing the reliability of the results they presented. Information regarding the concentration of the antibiotic administered was also neglected in the studies, with this data being of extreme importance, for example helping to explain why studies that used the same antibiotic presented differing results.

The discrepancies between the studies become clear when we take into consideration simple information such as age, weight, total number of animals and number of wounds per animal. Additionally, the absence of information regarding potential factors of confusion (for example, age, sex, concentration of medication) can lead to erroneous results. Therefore, our evaluation of the risk of bias and methodological quality show that many studies inadequately present their methodology, resulting in high risk of bias. Surprisingly, we did not find a direct relationship between the high methodological bias and the year of publication of the studies, that is, there has possibly been a systematic reproduction of methodological errors over the years, since the quality of the reports has not improved. These findings show the urgent need to develop controlled and randomized studies that aim to reduce biases in the selection, preparation and writing of scientific reports. Therefore, we expect that this revision will be used as a guide to improve reporting for future research into wound treatment with antibiotics.

Conclusion

The healing of cutaneous wounds has been widely investigated by researchers, being a fundamental area of research due to the considerable functional and aesthetic role of this tissue. When skin is injured, bacteria can infiltrate and colonize the surrounding tissue, which can lead to potentially fatal infections. Therefore, efficient treatment is necessary to control such pathological conditions.

An awareness of the effects of antibiotic treatments in healing of not intentionally infected wounds allows us to understand their influence on the flora and innate immunity of the skin, thereby, giving us a better understanding of the results expected from their use. In our revision, a large percentage of the treatments with antibiotics showed that they were effective in speeding up the healing process for wounds, given that a reduction in the infiltration of inflammatory cells, as well as an increase in the number of fibroblasts and extracellular matrix components and consequently a more rapid and effective closure of cutaneous wounds, was observed. However, the fragility of the current studies was evident, given that the majority presented high risk of bias, which impeded the reproducibility of most of the studies considered in this review.

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Supporting Information

S1 Table. Complete search strategy with search filters and number of studies recovered in databases PubMed-Medline and Scopus.

PubMed-MEDLINE - Search Filters	Number of records
#1 Antibiotic ("anti-bacterial agents"[MeSH Terms] OR "anti-bacterial agents"[TIAB] OR "antibiotic"[TIAB] OR "Anti Bacterial Agents"[TIAB] OR "Antibacterial Agents"[TIAB] OR "antibacterial"[TIAB] OR "Anti-Bacterial Compounds"[TIAB] OR "Anti Bacterial Compounds"[TIAB] OR "Bacteriocidal Agents"[TIAB] OR "Bacteriocides"[TIAB] OR "Anti-Mycobacterial Agents"[TIAB] OR "Anti Mycobacterial Agents"[TIAB] OR "Antimycobacterial Agents"[TIAB] OR "Antibiotics"[TIAB] OR "anti-biotic"[TIAB] OR "anti-infective agents"[MeSH Terms] OR "anti-infective agents"[TIAB] OR "Anti Infective Agents"[TIAB] OR "Antiinfective Agents"[TIAB] OR "antiinfective"[TIAB] OR "Microbicides"[TIAB] OR "Antimicrobial Agents"[TIAB] OR "Anti-Microbial Agents"[TIAB] OR "Anti Microbial Agents"[TIAB] OR "anti-microbial"[TIAB] OR "anti-infective"[TIAB] OR "antibiotic therapy"[TIAB])	**826371**
#2 Wound Healing ("Wound Healing"[MeSH terms] OR "Regeneration"[MeSH terms] OR "Wound Healing"[TIAB] OR "Regeneration"[TIAB])	**297911**
#3 Skin ("Skin"[MeSH terms] OR "Dermis"[MeSH terms] OR "Granulation Tissue"[MeSH terms] OR "Epidermis"[MeSH terms] OR "Keratinocytes"[MeSH terms] OR "Integumentary System"[MeSH terms] OR "Dermatology"[MeSH terms] OR "Dermoscopy"[MeSH terms] OR "Wounds and Injuries"[MeSH terms] OR "Fibrosis"[MeSH terms] OR "Skin injuries"[TIAB] OR "Skin fibrosis"[TIAB] OR "Skin scars"[TIAB] OR "Skin cicatriz"[TIAB] OR "Cicatrix"[MeSH terms] OR "cutaneous"[TIAB)	**1262536**
#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]	**6308321**

OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms])	
#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] 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] OR "amphibians"[TIAB] OR	**322580**

"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 "fitchew"[TIAB] OR "fitch"[TIAB] OR "beaver"[TIAB] OR "beavers"[TIAB] OR "jerboa"[TIAB] OR "jerboas"[TIAB] OR "capybara"[TIAB] OR "capybaras"[TIAB]] NOT "medline"[SUBSET])	
Combined search: ((((#5) OR #4) AND #3) AND #2) AND #1	**934**

SCOPUS - Search Filters	Retrieved records
#1 Antibiotic (TITLE-ABS-KEY ("anti-bacterial agents") OR TITLE-ABS-KEY (“anti-bacterial agents”) OR TITLE-ABS-KEY (“antibiotic”) OR TITLE-ABS-KEY (“Anti Bacterial Agents”) OR TITLE-ABS-KEY (“Antibacterial Agents”) OR TITLE-ABS-KEY (“antibacterial”) OR TITLE-ABS-KEY (“Anti-Bacterial Compounds”) OR TITLE-ABS-KEY (“Anti Bacterial Compounds”) OR TITLE-ABS-KEY (“Bacteriocidal Agents”) OR TITLE-ABS-KEY (“Bacteriocides”) OR TITLE-ABS-KEY (“Anti-Mycobacterial Agents”) OR TITLE-ABS-KEY (“Anti Mycobacterial Agents”) OR TITLE-ABS-KEY (“Antimycobacterial Agents”) OR TITLE-ABS-KEY (“Antibiotics”) OR TITLE-ABS-KEY (“anti-biotic”) OR TITLE-ABS-KEY (“anti-infective agents”) OR TITLE-ABS-KEY (“anti-infective agents”) OR TITLE-ABS-KEY (“Anti Infective Agents”) OR TITLE-ABS-KEY (“Antiinfective Agents”) OR TITLE-ABS-KEY (“antiinfective”) OR TITLE-ABS-KEY (“Microbicides”) OR TITLE-ABS-KEY (“Antimicrobial Agents”) OR TITLE-ABS-KEY (“Anti-Microbial Agents”) OR TITLE-ABS-KEY (“Anti Microbial”) Agents OR TITLE-ABS-KEY (“anti-microbial”) OR TITLE-ABS-KEY (“anti-infective”) OR TITLE-ABS-KEY (“antibiotic therapy”))	**777074**
#2 Wound Healing (TITLE-ABS-KEY (“wound healing”) OR TITLE-ABS-KEY (“Regeneration”))	**424226**
#3 Skin TITLE-ABS-KEY(Skin) OR TITLE-ABS-KEY(Dermis) OR TITLE-ABS-KEY(“Granulation Tissue”) OR TITLE-ABS-KEY(Epidermis) OR TITLE-ABS-KEY(Keratinocyte*) OR TITLE-ABS-KEY(Integumentary System) OR TITLE-ABS-KEY(Dermatology) OR TITLE-ABS-KEY(Dermoscopy) OR TITLE-ABS-KEY(Skin wounds) OR TITLE-ABS-KEY(Skin injuries) OR TITLE-ABS-KEY(Skin fibrosis) OR TITLE-ABS-KEY(Skin scar*) OR (Skin cicatrix) OR (cutaneous))injuries”) OR TITLE-ABS-KEY(“skin fibrosis”) OR TITLE-ABS-KEY(“skin scars”))	**1590396**
Combined search: #1 AND #2 AND #3 (Limited to experimental animals and English, Portuguese and Spanish language)	**602**

* In PubMed-Medline database, standardized animal filters were obtained in Hooijmans, et al. [25].

S2 Table. General characteristics of the experimental models used in all studies included in this systematic review.

Animal model: Rat								
Reference	**Country**	**Strain**	**Sex**	**Age**	**Weight**	**Total number**	**Ethics committee**	**Statistic test**
Leitch, et al. 1993 [27]	AUS	Sprague-Dawley	M	?	250–300 g	40	Yes	Yes
Heggers et al. 1995 [28]	USA	Sprague Dawley	?	?	?	60	?	Yes
Choi et al. 1999 [29]	KOR	Wistar	?	?	180—200 g	?	?	No
Muller et al. 2003 [30]	USA	Sprague Dawley	M	?	250–300 g	70	Yes	Yes
Kim, et al. 2008 [31]	KOR	Sprague-Dawley	M	?	250—300 g	?	?	No
Kim, et al. 2008 [32]	KOR	Sprague Dawley	M	?	250–300 g	?	?	No
Simpson, et al. 2008 [33]	USA	Sprague-Dawley	F	Adult (± 8 wk)	250–300 g	32	Yes	Yes
Hwang et al. 2010 [34]	KOR	Sprague Dawley	?	?	250–280 g	10	Yes	Yes
Lin et al. 2010 [35]	TWN	Sprague Dawley	M	8 wk	250–300 g	10	Yes	Yes
Huang et al. 2012 [36]	TUR	Sprague Dawley	F	4 mo	200–250 g	10	Yes	Yes
Gurel et al. 2013 [37]	IND	Sprague-Dawley	F	?	220–250 g	?	Yes	Yes
Mittal and Kumar 2014 [38]	IND	Wistar	M	?	?	?	?	No
Princely et al. 2015 [39]	CHN	Sprague Dawley	?	?	200–250 g	12	Yes	Yes
Fu et al. 2016 [40]	CHN	Sprague-Dawley	M	?	220–250 g	35	Yes	No
Animal model: Pig								
Reference	**Country**	**Strain**	**Sex**	**Age**	**Weight**	**Total number**	**Ethics committee**	**Statistic test**
Geronemus, et al. 1979 [42]	USA	?	?	Young (± 3 yr)	5.5–9.1 kg	17	?	Yes
Watcher and Wheeland 1989 [43]	USA	Minipig	?	Young (± 3 yr)	10.5/ 13.0 kg	2	?	Yes
Singer, et al. 1999 [44]	USA	Yorkshire	F	?	20–30 kg	3	Yes	Yes
Faucher, et al. 2010 [45]	USA	Yucatan	?	?	18kg	8	Yes	Yes
Theunissen et al. 2016 [46]	ZAF	?	F	?	5–20 kg	16	Yes	Yes
Animal model: Mice								
Reference	**Country**	**Strain/ Animal**	**Sex**	**Age**	**Weight**	**Total number**	**Ethics committee**	**Statistic test**
Hebda et al. 2003 [47]	USA	FVB	?	?	?	16/24	Yes	Yes
Zhang et al. 2015 [48]	TWN	C57BL/6J	M	8–10 wk	?	8	Yes	Yes
Tummalapalli et al. 2016 [49]	IND	C57BL/6J	M	10 wk	20–25 g	25	Yes	Yes
Kataria et al. 2014 [50]	IND	?	M	?	1.5–3 kg	12	Yes	No
Qian, et al. 2017 [51]	USA	New Zealand	F	Adult (± 6 mo)	3–5 kg	24	Yes	Yes

Berry and Sullins 2003 [52]	USA	Thoroughbreds, Warmblood and Quarter Horse	M	5–11 yr	527–645 kg	6	Yes	Yes
Edwards-Milewski et al. 2016 [53]	USA	Quarter Horses and Thoroughbreds	M/F	6–23 yr	402–581 kg	7	Yes	Yes

AUS = Australia, CHN = China, d = day, F = female, IND = India, KOR = South Korea, M = male, mo = month, TUR = Turkey, TWN = Taiwan, USA = United States of America, wk = week, yr = year, ZAF = South Africa.

S3 Table. General characteristics of the wounds treated with antibiotics used in all studies included in this systematic review.

Wound						
Animal model: Rat						
Reference	**Asepxis**	**Biopsia day**	**Realization**	**Size**	**Number for animal**	**Anesthesia (drug and dose)**
Leitch, et al. 1993 [27]	?	?	?	2.25 cm^2	4	Iso (?)
Heggers et al. 1995 [28]	?	?	?	1.5 cm^2	4	?
Choi et al. 1999 [29]	PI	5/12	?	1 cm (Diam)	?	Thiopental (?)
Muller et al. 2003 [30]	?	NA	?	2.25 cm^2	4	Pentobarbital (?)
Kim, et al. 2008 [31]	EtOH 70%	5/15	?	2.25 cm^2	2	Tilet + Zolaz (?)/ Xyl (?)
Kim, et al. 2008 [32]	EtOH 70%	5/ 10/ 15	?	2.25 cm^2	2	Tilet (125,0 mg) + Zolaz (125,0 mg) / Xyl (20 mg/mL)
Simpson, et al. 2008 [33]	CHX 4%/ sal	4/ 8/ 14	?	8 mm (Diam)	2	Iso (1.25–2%)
Hwang et al. 2010 [34]	EtOH 70%	15	?	2.25 cm^2	2	Tilet (125,0 mg) + Zolaz (125,0 mg)
Lin et al. 2010 [35]	EtOH 70%	NA	?	1 cm^2	2	Ket (90 mg/kg)/ Xyl (10 mg/kg)
Huang et al. 2012 [36]	EtOH 70%	2/ 4/ 8/ 12	?	1 cm^2	?	Ket (90 mg/kg) + Xyl (10 mg/kg)
Gurel et al. 2013 [37]	Sal	4/ 7/ 14/ 21	Punch biopsy/ scalpel	5 mm	12	Ket (70 mg/kg) + Cpz (3 mg/kg)
Mittal and Kumar 2014 [38]	EtOH 70%	3/ 7/ 14/ 28/ 45	?	2 cm (Diam)	?	Ket (80 mg/kg) +Xyl (5 mg/kg)
Princely et al. 2015 [39]	?	NA	Scissor	2 cm, 2 mm deep	1	Ket (50 mg/kg)
Fu et al. 2016 [40]	EtOH 75%	8/ 14	Scissor	10 mm (Diam)	4	Chloral hydrate (480 mg/kg)
Li et al. 2017 [41]	?	21	?	9 cm^2	1	Pentobarbitone (50 mg/kg)
Animal model: Pig						
Reference	**Asepxis**	**Biopsia day**	**Realization**	**Size**	**Number for animal**	**Anesthesia (drug and dose)**
Geronemus, et al. 1979 [42]	?	2–7	Dermatome	0.7 cm^2, 0.3 mm deep	150	Pentobarbital (15 mg/kg)
Watcher and Wheeland 1989 [43]	PI	NA	Scalpel	4 cm^2	20	Ket (10 mg/kg) + Halo (?)
Singer, et al. 1999 [44]	PI/ sal	7/ 14/ 30	Punch biopsy	4 mm	36/45	Tilet + Zolaz (5 mg/kg) / Iso (0.5-2.5%)
Faucher, et al. 2010 [45]	PI	7/ 9/ 14/ 21	Dermatome	0.055/ 0.076 cm	2	Tilet + Zolaz (4.4 mg/kg) + Xyl (2.2 mg/kg)/ Iso
Theunissen et al. 2016 [46]	No	3/ 7/ 28	Scalpel	4 cm^2	16	Halo (?)
Animal model: Mice						

Reference	Asepxis	Biopsia day	Realization	Size	Number for animal	Anesthesia (drug and dose)
Hebda et al. 2003 [47]	?	7/ 14/ 21	Dermatome/ scissor	0.7 cm^2, 0.2–0.3 mm deep/ 1cm (Diam)	1	Halo (?)
Zhang et al. 2015 [48]	?	5	Punch biopsy	8 mm	?	Pentobarbital (?)
Tummalapalli et al. 2016 [49]	?	21	Punch biopsy	8 mm	1	Iso (?)
Animal model: Rabbit						
Reference	**Asepxis**	**Biopsia day**	**Realization**	**Size**	**Number for animal**	**Anesthesia (drug and dose)**
Kataria et al. 2014 [50]	?	5/ 15	Scalpel	4 cm^2, 0.5 cm deep	1	Ket (75 mg/kg)
Qian, et al. 2017 [51]	PI/ EtOH 70%	7/ 28	?	7mm (Diam)	6	Ket (22.5 mg/kg) + Xyl (3.5 mg/kg)/ Lid (1%) + Epi
Animal model: Horse						
Reference	**Asepxis**	**Biopsia day**	**Realization**	**Size**	**Number for animal**	**Anesthesia (drug and dose)**
Berry and Sullins 2003 [52]	CHX	NA	Scalpel	6.25 cm^2	6	Ket (2.2 mg/kg)/ Xyl (500 mg) + Ket (2 mg) + guaifenesin (50 g)
Edwards-Milewski et al. 2016 [53]	CHX 4% / sal/ EtOH	2/ 5/ 13	Scalpel	6.25 cm^2	4	Detomidine (0.01 mg/kg) + butorphanol tartrate (0.04 mg/kg)/ mepivacaine (2%)

CHX = chlorhexidine, Cpz = Chlorpromazine, Diam = diameter, Epi = epinephrine, EtOH = ethanol, Halo = Halothane, Iso = Isoflurane, Ket = Ketamine, Lid = lidocaine, NA = Not applicable, PI= povidone-iodine, Sal = saline solution, Tilet = Tiletamine, Xyl = xylazine, Zolaz = Zolazep.

S4 Table. Guide for relevant information in studies with antibiotic therapy and wound healing.

General characteristics of experiments	General characteristics of experimental models	Characteristics of antibiotic treatment	Wound characteristics
1. Statistical procedure; 2. Sample size (n) for each analysis; 3. Animal ethics committee approval; 4. Appropriate control group (same formulation given for experimental groups, except antibiotic).	1. Animal model; 2. Strain; 3. Animals age; 4. Average weight of animals; 5. Animals sex; 6. Animals amount per group.	1. Drug; 2. Pharmaceutical form; 3. Drug concentration; 4. Administered dose; 5. Route of administration; 6. Administration number per period; 7. Treatment duration.	1. Material used to shave the animal; 2. Information about skin assepsis before surgical procedure; 3. Anesthetic drug; 4. Object used to make the would; 5. Wound area; 6. Would number per animal; 7. Drug used after surgical procedure; 8. Occlusion or lack of wound occlusion; Wound area assessment periods; 9. Biopsy days;

Important points that should be reported in antibiotic therapy studies to improve study quality and reports

3 ARTIGO II

Doxycycline hyclato stimulates the antioxidant defenses and accelerates the repair of skin wounds in rats

Abstract

We investigated the effect of doxycycline hyclate (Dx), a broad-spectrum antibiotic inhibitor of matrix metalloproteinase (MMPs), on the viability of macrophages in vitro and in vivo on cutaneous repair in Wistar rats. Male animals (n=15) were kept in cages with food and water ad libitum this study was approved by the Guiding Principles in the Use of Animal Ethics Committee (protocol: 72/2017). After applying the anesthetic, three skin wounds (12 mm diameter) were created on the animals' back, and they were randomized into three groups: C: received distilled water (control); Dx1: doxycycline hyclate (10 mg/kg/day); Dx2: doxycycline hyclate (30 mg/kg/day). The applications were carried out daily for up to 21 days, and tissues from different wounds were removed every 7 days. The wounds were collected for histopathological and biochemical analysis and metalloproteinase expression (MMP-10). Our findings showed for in vitro analysis Dx led to the proliferation of macrophages. In vivo analysis showed an increase of cells and extracellular matrix compounds (collagen and elastin) in cutaneous lesions, and Dx showed a reduction in oxidative markers such as protein carbonyls, malondialdehyde, and nitric oxide. In addition, Dx caused an increase in antioxidant enzymes (catalase and superoxide dismutase) and a reduction in Matrix Metalloproteinases (MMP-10). Our findings indicated that Dx produced a better healing pattern with complete reepithelization. In addition, proved to have a positive effect on the morphology of the scar tissue, as well as an antioxidant stimulating effect during the repairing of the skin wounds in the rats.

Keywords: Metalloproteinase, oxidative stress, cicatrization, extracellular matrix, antibiotic

INTRODUCTION

The skin is a complex organ that serves as a barrier to protect the body from the external environment [1]. However, different aggressive agents, such as a trauma and microorganisms, can affect the structure and functions of this organ. In the case of a lesion, there is an exposure of subcutaneous tissue providing a humid and nutritious environment for the proliferation and microbial colonization [2]. An infected cutaneous wound increases the risks of chronification, reduces the quality of life and causes a high mortality rate of patients [3]. Skin wounds represent a serious health problem worldwide, and are frequently associated with high costs and inefficient treatments whit limited efficiency [4]. The therapies available today, aim to improve the healing of wounds by promoting their rapid closure. However, the control of infections is generally neglected [5]. As a result, it is desirable to develop therapeutic interventions that control the infection and increase the cutaneous repair.

The repairing of cutaneous wounds is a process that involves a complex interaction between cells, extracellular matrix, blood vessels and tissue growth factors. Furthermore, the process is separated into the phases of inflammation, proliferation (granulation) and tissue remodeling [6]. During the inflammation phase, there is a migration of leucocytes to the injured site. During the proliferative phase, there is a multiplication of keratinocytes, fibroblasts and endothelial cells, resulting in the formation of granulation tissue, which is also rich in vessels and collagen type III [7]. The next phase is characterized by remodeling and maturation of the tissue, in which collagen III is substituted for collagen I, because it is stronger and more resistant to mechanical forces [8,9].

The skin healing process is known as acute or chronic according to its duration and nature [7]. During a chronification process, neutrophils and macrophages release cytokines and chemokines, which attract more cells in the location of the inflammation, and promote oxidative stress in the repairing tissue [10]. The excess of pro-inflammatory mediators promotes the increase of the peroxide of hydrogen (H_2O_2) and oxide nitric content which they accelerate the peroxidation of the lipids and proteins [11]. The pro-oxidant mediations cause damage to the cutaneous tissue and delay the wound healing process. There is usually a decrease in the synthesis and expression of the antioxidant enzymes such as superoxide, glutathione and catalase, impairing the healing environment [10,12].

In general, a desirable repair process results in a balance in the normal process of synthesis and degradation of extracellular matrix components, especially collagen [13]. Matrix metalloproteinases (MMP), play an essential role in extracellular matrix (ECM) remodeling, by degrading collagen and non-collagenous elements, such as glycosaminoglycans, proteoglycans,

cytokines, growth factors and their receptors [14]. However, the overexpression of MMP may lead to uncontrolled degradation of ECM and delay the wound healing process [15,16]. Thus, MMPs modulatory drugs may cause an important impact on cutaneous tissue repair, with a particular effect on tissue inflammation and maturation. In this context, doxycycline (Dx) has already been described as an inhibitor of MMPs activity [17], and its usage has previously been proven in the modulation of tissue levels of collagen in cutaneous repair [18], stimulating collagen deposition. In addition, Dx showed to be an important tool to inhibit the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-6 and IL-8 [19]. Based on this, it is possible that the Dx may affect the cutaneous healing process. Although Dx is effective in treating various disorders [20-22], little is known about the role of Dx in skin tissue repair. Therefore, this study evaluated the effect of Dx on the viability of macrophages in vitro and used an experimental model to understand the effect of Dx in the healing of wounds in rats.

MATERIALS AND METHODS

Cell Viability Assay

Cell viability for RAW 264.7 macrophages was evaluated by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [23,24]. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin in a humidified 5% CO_2 37°C incubator. To evaluate the effect of Dx on cell viability, RAW 264.7 macrophages were seeded into 96-well plates at a density of 1×10^5 cells/well in 200 μL medium. After 24 h, the various concentrations of Dx (10, 30, 100, and 300 μg/mL) were added to media, and the incubation continued for the next 24 h at 37°C and 5% CO_2. The control (100 % of growth) were made with cells cultured in medium only. MTT solution was added to each well and the cells were further incubated for 2 h at 37°C. MTT formazan generated during incubation was dissolved in DMSO, and the absorbance was measured at 570 nm. For each sample, the result was expressed as the percentage absorbance in relation to the control group.

Animals

Fifteen healthy three month old male Wistar rats (Rattus norvegicus) (339.16 ± 16.25 g), were obtained from the Central House of the health and Biological Sciences Center, Federal University of Viçosa. These animals were randomly allocated in individual cages that were

cleaned daily and under controlled environmental conditions (temperature: 22 ± 2 °C, humidity: 60–70%, and light/dark cycle: 12/12 h). Commercial food and water were provided ad libitum. All the experiments were approved by the Animal Ethics Committee of the Federal University of Viçosa (registration nº 72/2017).

Procedure of surgical wounds

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (70 mg/ kg). After anesthesia, dorsolateral shaving of the animals was performed, and the area was cleaned with 70% alcohol. Three circular secondary intention wounds of 12 mm diameter were made in the dorso-lateral region of each rat, until exposure of the dorsal muscular fascia using surgical scissors. The area of the wound was marked using violet crystal and measured with an analog caliper (Mitutoyo Sul Americana Ltda®, São Paulo, Brasil) [25]. No analgesic was administered after the surgical procedure, since the application of drugs can alter cell migration and proliferation, compromising the skin repairing process. Tissue samples were obtained from different wounds at 7, 14 and 21 days for histological and biochemical analysis, as shown in the Figure 1.

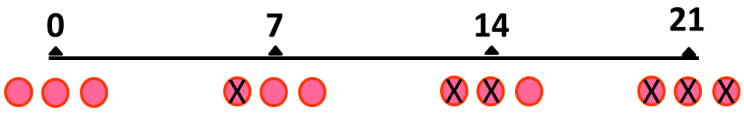

Figure 1. Schematic representation of the period for obtaining tissue samples from different wounds on days 7, 14 and 21.

Experimental design

The animals were randomized into three groups with five animals in each group: C (distilled water, control); Dx1 (doxycycline 10 mg/kg/day) and Dx2 (doxycycline 30 mg/kg/day). Doses were chosen based on literature studies. The administration of the treatments was performed by gavage for 21 days. After this period, the animals were euthanized by cardiac puncture and exsanguination after the anesthetic procedure.

Calculation of the area and the rate of wound contraction

The area and rate of contraction of the third wound was evaluated every 7 days using images scanned with 320×240 pixels (24 bits/pixel) obtained by the smartphone Asus Zenfone 2 ZE551ML (ASUS, São Paulo, Brazil). The wound area was calculated by the formula A= Π

× radius $(r)^2$, where r is the radius. The wound contraction index (WCI) was calculated by the ratio: initial wound area (Ao) — area on a given day (Ai) / initial wound area (Ao) × 100 [26].

Histological analysis

The samples collected from the wounds, with tissue from the center of the lesion and part of the tissue adjacent to the edges of the lesions, were fixed in Karnovsky solution, dehydrated in ethyl alcohol, diaphanized in xylol and immersed in paraffin. Histological sections (4μm thick) were obtained on rotary microtome (Leica Multicut® 2045, Reichert-Jung Products, Germany). We used 1 of every 20 sections to avoid repeating the analysis of the same histological area. Sections were stained with hematoxylin and eosin (HE) for analysis of the fibroblasts and blood vessels [25]. Furthermore, the samples were stained with Sirius Red for the analysis of collagen fibers type I and III [27], toluidine blue was used to identify mast cells [28], and Verhoeff was used to differentiate elastic fibers [12]. The slides were visualized and captured in a BX601 light microscope (Olympus, São Paulo, Brazil) coupled to a QColor-31 digital camera (Olympus, São Paulo, Brazil). Five images were selected at random using a 20 objective lens. For this analysis, a grid containing 256 points within a standard test area (AT) of 73 x 10^3 μm^2 was superimposed over each image. The stereological parameters of volumetric density (Vv) were calculated by counting the points that occurred over cels, blood vessels, type I and type III collagen and elastic fibers, using the ratio: Vv= PP/PT. PP is the number of points occurring over the structures of interest and PT is the total number of points on the test system [25,29]. Collagen fibers were analyzed according to the different properties of birefringence, as thick collagen fibers (type I) appear in shades of bright colors ranging from red to yellow, whereas thin reticular fibers (collagen type III) appear bright green under polarization [25,30]. Mast cells were analyzed using a 40x objective lens, 10 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 according to the relation QA= Σ mast cells / TA [31].

Biochemical analysis

Tissue fragments were collected from each wound and immediately frozen in liquid nitrogen (-196°C), and stored in a freezer at −80° C. The samples were homogenized in phosphate buffered saline (PBS), 100 mg sample and 1 mL PBS, and centrifuged for 5 minutes at 10000g (12000 rpm) under refrigeration at 5º C [12].

Hydrogen peroxide production

The Hydrogen peroxide (H_2O_2) production was measured in supernatants of homogenates of tissues. 50 µL of supernatants were incubated with 50 µL of αPhenylenediamine dihydrochloride (OPD), and an equal volume of peroxidase type II 15 mmol/L. The conversion of absorbance into micromolar concentrations of H_2O_2 was calculated from a standard curve using a known concentration of H_2O_2. The results were expressed as µmol/L [32].

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Nitric oxide analysis

Nitric oxide (NO) was indirectly quantified through the detection of nitrite/nitrate (NO_2^- /NO_3^-) levels by the standard Griess reaction [33]. 50 µL of supernatants were incubated with an equal volume of Griess reagent, (1% sulfanilamida, 0,1% N-(1-Naftil) etilenodiamina e 2,5% H_3PO_4) and kept at room temperature for 10 minutes. The conversion of absorbance into micromolar concentrations of NO was obtained from a sodium nitrite standard curve (0–125 µM) and expressed as NO concentrations (µmol x L^{-1}).

Determination of malondialdehyde

Lipid peroxidation (LPO) was estimated according to the total malondialdehyde levels (MDA) [34]. The concentration of MDA was determined by using the standard curve of known concentrations of 1, 1, 3, 3-tetramethoxypropane (TMPO). The results were expressed as µmol x L^{-1} per mg of protein. bradford

Protein oxidation

Protein carbonyl content was measured using 2, 4-dinitrophenylhydrazine (DNPH) [35], based on the carbonyl groups reaction with DNPH. The pellets resulting from the tissue homogenates from previous extractions were used for quantification. The results were expressed as nmol per mL of protein.

Superoxide dismutase activity

The activity of superoxide dismutase (SOD) was determined by the method using the reduction of the superoxide (O-2) and hydrogen peroxide, thereby decreasing the auto-oxidation of pyrogallol [36]. SOD activity was calculated as units per milligram of protein, with one unit (U) of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%.

Catalase activity

The catalase (CAT) activity was evaluated according to the method described by Aebi (1984) [37], through measuring the rate of decomposition of hydrogen peroxide. One unit of CAT activity was calculated using the amount of enzymes that decompose one mmol H_2O_2 for 1 min. The results were expressed as units of catalase/milligram of protein.

Glutathione S-transferase activity

The glutathione S-transferase (GST) activity was measured using the method of Habig et al. (1974) [38]. Glutathione S-transferase activity was analyzed according to the formation of glutathione-conjugated 2,4- dinitrochlorobenzene (CDNB). One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of one μmol of product/min/mL. GST activity was expressed as U per milligram of protein.

Evaluation of MMP-10 cutaneous activity

For the evaluation of MMP-10 activity, 200-mg samples of the skin were homogenized in 1ml of 5 mM Tris-HCl (pH 7.4) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3. After centrifugation at 10,000 xg for 30 min, the supernatant was collected for analysis of MMP activity. For this, an ELISA commercial immunoenzymatic kit was used according to the manufacturer's instructions (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, Calif., USA). The results were expressed as mean and standard deviation (mean ± SD) and a one-way ANOVA variance analysis was done followed by the Student-Newman-Keuls parametric test. Statistical significance was established at $P < 0.05$.

RESULTS

Cell Viability Assay

The effect of Dx on macrophages viability is shown in Figure 1. No cytotoxicity was observed after exposing the macrophage cells to Dx. Furthermore, the highest cell proliferation was observed after an incubation of 100 (182,03 ± 6,57) and 300 μg/ml (253,05 ± 30,92) in a dose-dependent manner (Figure 2 (a) e (b)).

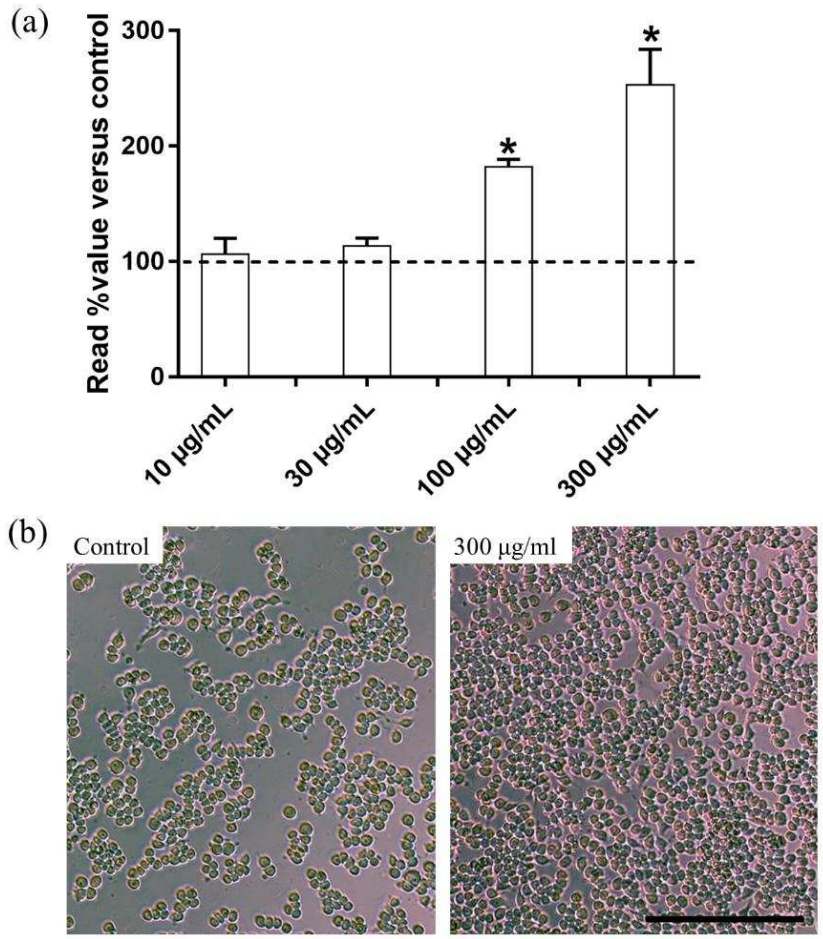

Figure 2. Effects of doxycycline (Dx) on cell viability. (a) RAW 264.7 macrophages were treated with various doses of Dx (10, 30, 100, and 300 µg/mL) for 24h. (b)representative photomicrographs showing cells cultured in only medium (control group) and Dx added to the medium (300 µg/mL). Bar = 200 µm. Results are presented as the percentage absorbance of the control group. Data are expressed as the means ± SD, * vs. the control group.

Wound area and contraction index

The wound area was lower on the 7th, 14th and 21st day in the Dx1 (10 mg/kg) group, when compared to the control group, as well as on the 14th day in the Dx2 (30 mg/kg) group, in relation to the control group. The wound contraction index was higher in the Dx1 and Dx2 groups, when compared to control on the 21st day (Table 1).

Tabela 1. Area (mm^2) and rate of wound contraction (mm^2/day) in rats treated with doxycicline hyclate after 7, 14 and 21 days of treatment

Day	Area/contraction	Control	Dx1	Dx2
0	A(mm2)	13.63 ± 0.48	11.90 ± 0.82	12.96 ± 0.05
	WCI (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7	A(mm2)	**11.00 ± 1.35^{a}**	**8.70 ± 1.08^{b}**	**10.50 ± 1.54^{ab}**
	WCI (%)	19.36 ± 8.22	27.34 ± 11.30	19.00 ± 11.74
14	A(mm2)	**6.63 ± 1.89^{a}**	**4.4 ± 1.08^{b}**	**5.20 ± 2.17^{b}**
	WCI (%)	51.04 ± 15.44	63.16 ± 8.01	59.90 ± 16.66
21	A(mm2)	**3.23 ± 1.58^{a}**	**1.5 ± 0.50^{b}**	**2.20 ± 0.76^{ab}**
	WCI (%)	**76.42 ± 11.06^{a}**	**87.34 ± 4.37^{b}**	**83.01 ± 5.87^{b}**

Date are reported as mean± S.D: A= Wound area: WCI= wound contraction index, Dx1= Doxycycline hyclate (10mg/kg), Dx2= Doxycycline hyclate (30mg/kg). ^{a,b} Different letters in lines indicate statistically significant differences between groups ($p<0.05$), Kruskal–Wallis test.

Histopathologicals results

On day 7, the proportion of cells was higher in the Dx2 group, when compared to other groups. On the same day, Dx1 showed a higher proportion of cells, when compared to the control group. On day 14, Dx2 presented a higher proportion of cells, when compared to control group (Figure 3 [a]). In relation to the proportion of blood vessels, the groups Dx1 and Dx2, showed an increase when compared to the control group on day 7. However, on day 14, the proportion of vessels were reduced in the Dx1 group, when compared to the control group. On the 21st day, Dx1 showed a reduction in blood vessels when compared to the control group and Dx2 groups (Figure 3 [a]). The representative distribution of cells and blood vessels in the scar tissue of the different groups is shown in Figure 3 (b).

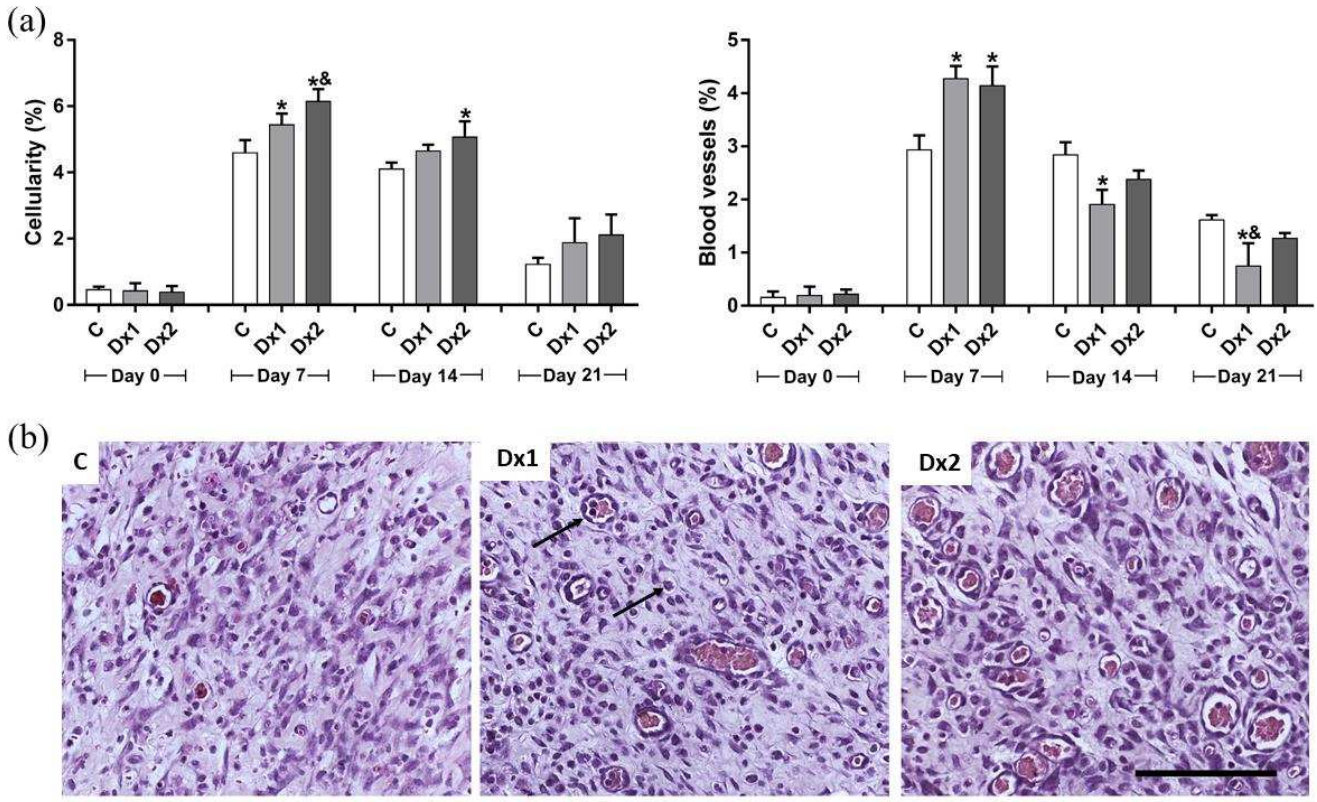

Figure 3. (a) Proportion of cell nucleus and blood vessels and (b) representative photomicrographs showing cells and blood vessels distribution in the scar tissue of rats treated with Doxycycline hyclate on day seven, bar = 100 µm. C= control, Dx1= Doxycycline hyclate (10mg/kg), Dx2= Doxycycline hyclate (30mg/kg). Data represented as mean ± SD, * vs. Control and & vs. Dx1 or Dx2 (Newman-Keuls test, $p<0.05$).

The number of mast cells on the 7th day was higher in the Dx2 group, when compared to other groups. In addition, Dx1 group presented the highest number of cells when compared to the control group (Figure 4 [a]).

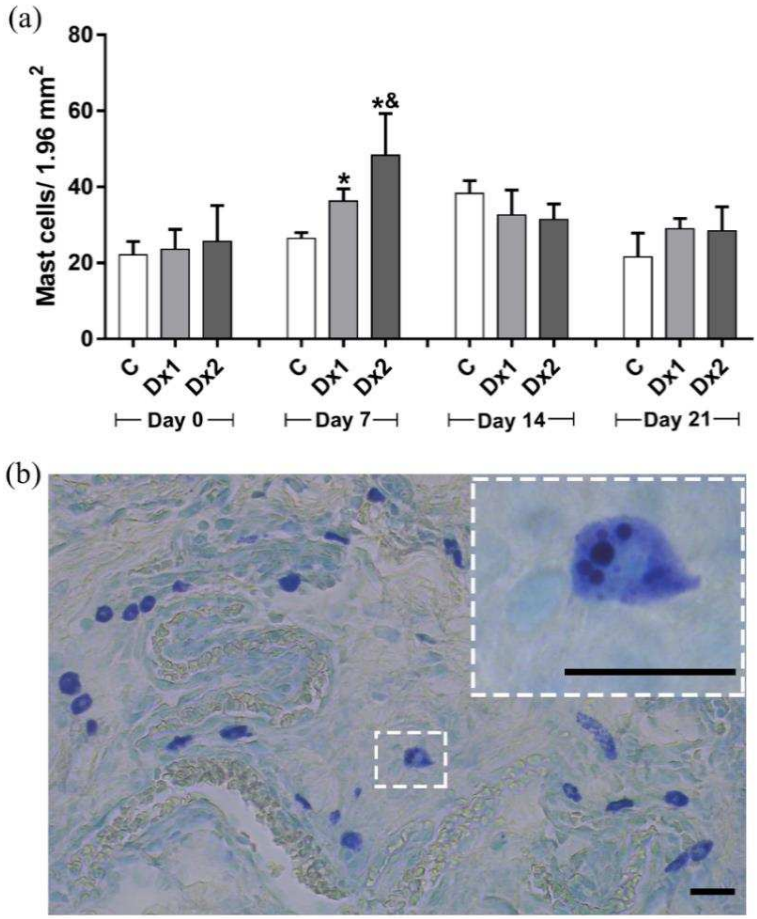

Figure 4. (a) Number of mast cells and (b) representative photomicrograph showing mast cells distribution in the scar tissue of rats treated with Doxycycline hyclate on day seven, group Dx2, bar = 50 µm. C= control, Dx1= Doxycycline hyclate (10 mg/kg), Dx2= Doxycycline hyclate (30 mg/kg). Data represented as mean ± SD, * vs. Control and & vs. Dx1 or Dx2 (Newman-Keuls test, $p<0.05$).

A higher proportion of type I collagen fibers were observed in the groups treated with Dx1 and Dx2 on the 21st day, when compared to the control group. The type III collagen fibers were reduced in Dx1 and Dx2 on day 21 in comparison to the control group (Figure 5 [a]). The representative distribution of type I and type III fibers in the scar tissue of the different groups is shown in Figure 5 (b). On day 14 and 21, the elastic fibers increased in the Dx2 group, when compared to the other groups Figure 5 [c]). Representative distribution of elastic fibers in the scar tissue of the different groups is shown in Figure 5 (d).

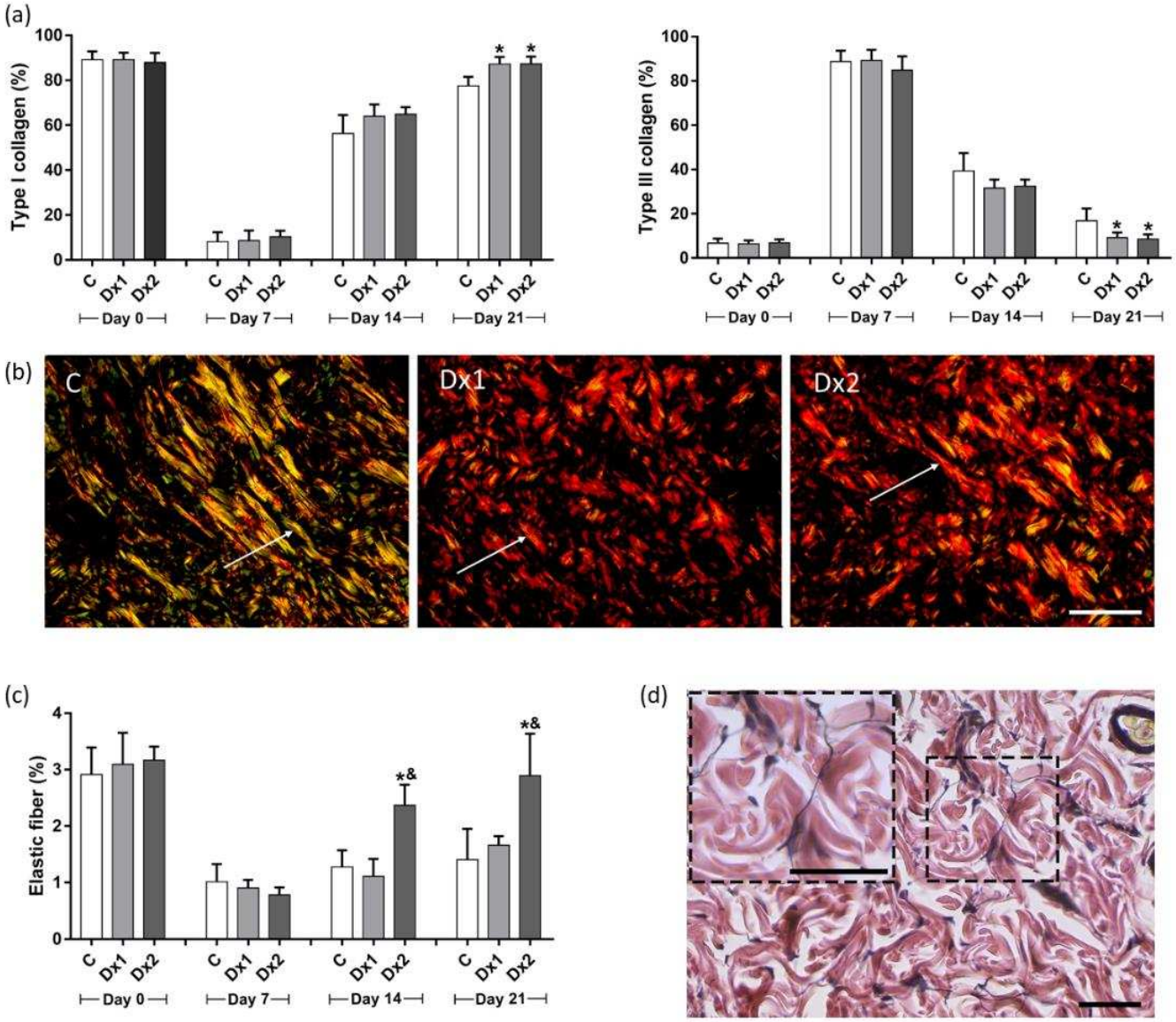

Figure 5. (a) Proportion of type I and III collagen fibers, (b) representative photomicrographs showing collagen fibers distribution on the 21st, bar = 100 µm, (c) proportion of elastic fibers, and (d) representative photomicrograph showing elastic fibers distribution in group Dx2 on day 21, bar =50 µm, in the scar tissue of rats treated with Doxycycline hyclate. C= control, Dx1= Doxycycline hyclate (10mg/kg), Dx2= Doxycycline hyclate (30mg/kg). Data represented as mean ± SD, * vs. Control and & vs. Dx1 or Dx2 (Newman-Keuls test, $p<0.05$).

Biochemical results

Oxidative stress markers

On day 7, H_2O_2 levels were higher in the groups treated with Dx1 and Dx2, in relation to the control group. On day 14, Dx2 showed lower levels of H_2O_2, in relation to the control

and Dx1 groups. On day 21, Dx2 showed lower levels of H_2O_2, when compared to the control group (Figure 6 [a]). The concentrations of nitrite and nitrate (NO_2^-/NO_3^-), were lower in Dx1 and Dx2 groups, when compared to the control group on day 14 (Figure 6 [b]). On day 7, malondialdehyde (MDA) levels were lower in the groups treated with Dx1 and Dx2 (Figure 6 [c]). On the other hand, the concentration of carbonyl proteins (PC) in the Dx1 group showed lower levels in relation to the control group, on day 21 (Figure 6 [d]).

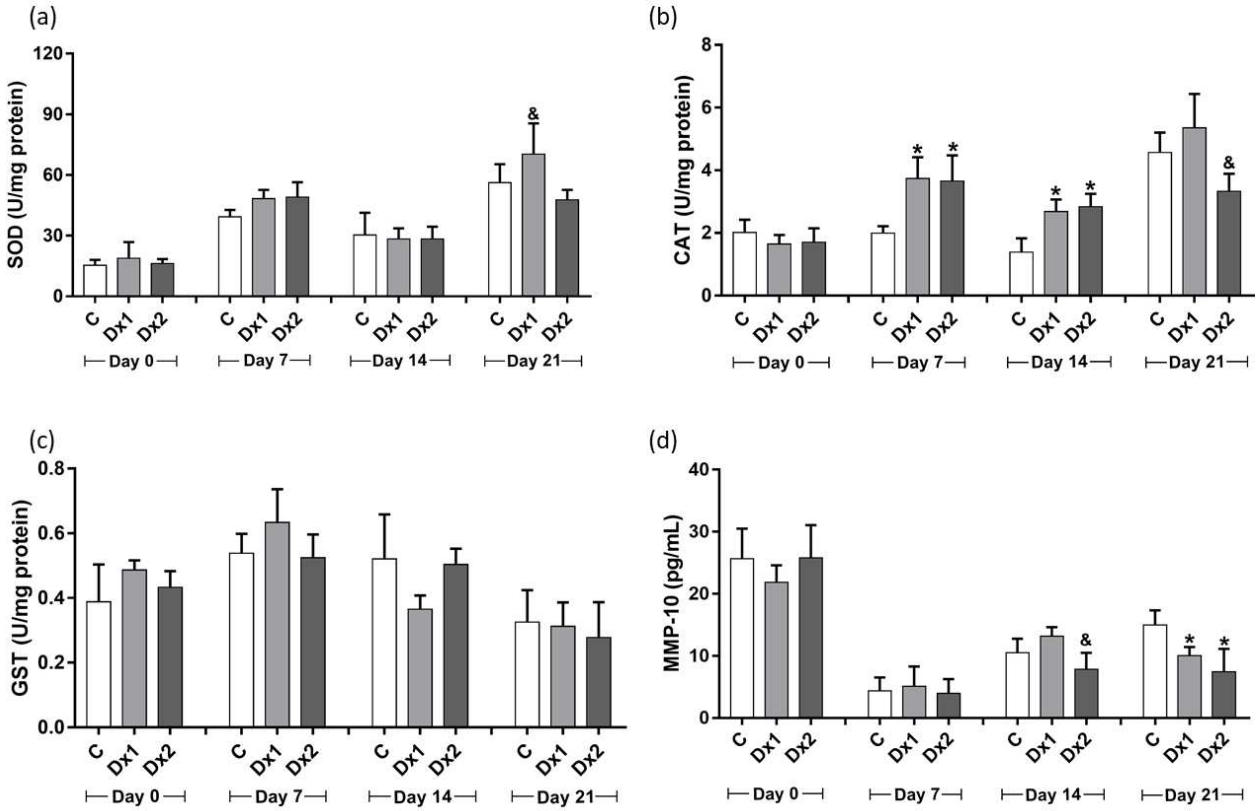

Figure 6. Levels of (a) hydrogen peroxide (H_2O_2), (b) nitrite and nitrate (NO_2^-/NO_3^-), (c) malondialdehyde (MDA), and (d) carbonylated proteins (CP) in the scar tissue of rats treated with Doxycycline hyclate. C= control, Dx1= Doxycycline hyclate (10mg/kg), Dx2= Doxycycline hyclate (30mg/kg). Data represented as mean ± SD, * vs. Control and & vs. Dx1 or Dx2 (Newman-Keuls test, $p<0.05$).

Antioxidants enzymes and metalloproteinase-10

The Superoxide dismutase (SOD) activity was higher in the Dx1 group, on day 21, when compared to the Dx2 group (Figure 7 [a]). The Catalase (CAT) activity was higher on days 7 and 14, in Dx1 and Dx2 groups when compared to the control. On day 21, the CAT activity was lower in Dx2, in relation to the Dx1 group (Figure 7 [b]). The Glutathione S- transferase (GST) values were not significantly different over the trial period (Figure 7 [c]). On day 14, the

MMP-10 levels were lower in group Dx2 compared to Dx1. On day 21, both groups treated with Dx had lower levels of MMP-10 when compared to the control group (Figure 7 [d]).

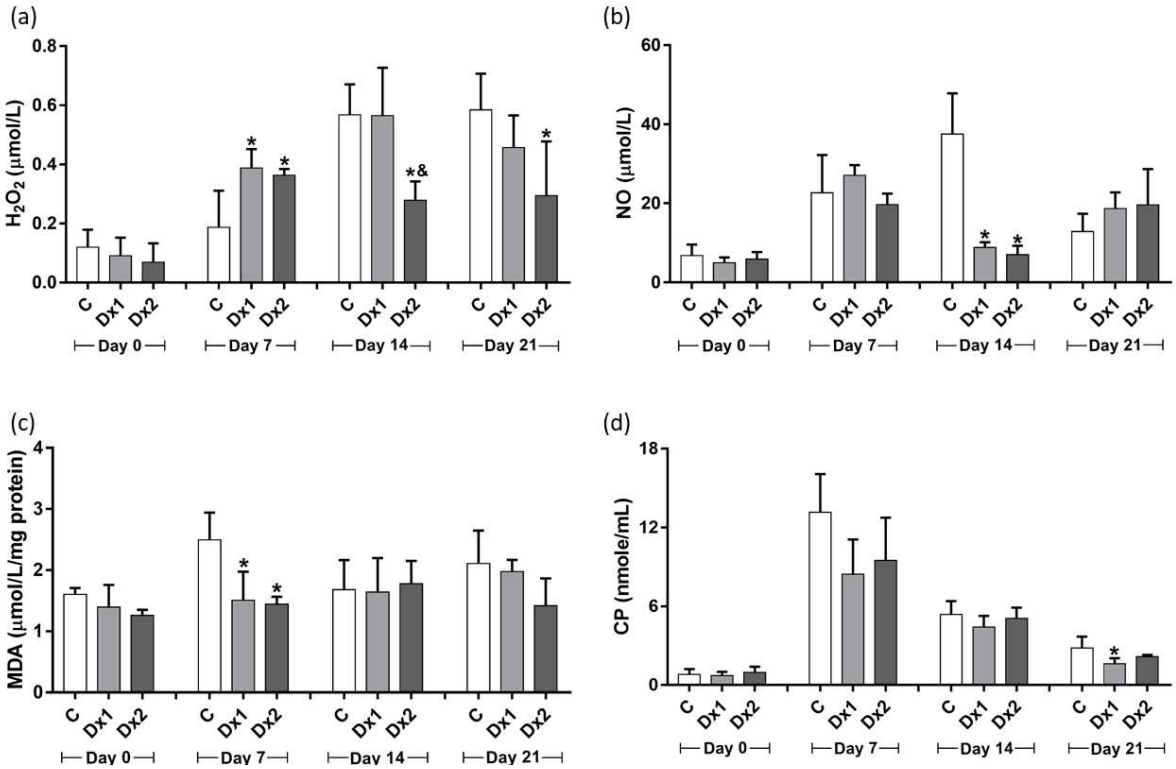

Figure 7. Levels of (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) glutathione S-transferase (GST), and (d) metalloproteinase-10 (MMP-10) in the scar tissue of rats treated with Doxycycline hyclate. C= control, Dx1= Doxycycline hyclate (10mg/kg), Dx2= Doxycycline hyclate (30mg/kg). Data represented as mean ± SD, * vs. Control and & vs. Dx1 or Dx2 (Newman-Keuls test, $p<0.05$).

DISCUSSION

Doxycycline constitutes a large group of broad-spectrum antibiotics derived from tetracycline [39]. Studies have suggested that Dx has therapeutic activities that are not related to its antimicrobial activity [40,41]. The present study used an integrated biochemical, morphological and oxidative analysis to evaluate the effect Dx has when administrated orally in Wistar rats, to repair their skin. We observed that Dx was effective for a complete wound closure, as well as, increasing the rate of contraction. This effect possibly is associated with antimicrobial, anti-inflammatory and the antioxidant capacity of Dx [40,42]. These results showed that Dx has beneficial action for the treatment of skin lesions, however, studies investigating the action of this drug on the cutaneous repair are still scarce and limited.

Due to its potent bacteriostatic properties, Dx is an effect antibiotic to treat diseases such as syphilis [20], periodontal diseases [21], pneumonia [43], and cholera [44]. Although the effect of Dx on cutaneous repair is still poorly explored, this drug promotes an increase in the collagen deposition [45], stimulates tissue re-epithelization [17] and favors elimination reactive oxygen species, thereby preventing or reducing pathological tissue destruction [46]. In addition, we observed that after the treatment with Dx, there was an increase of macrophages in the in vitro analysis, thus Dx seems to also contribute to immunoregulatory activity. Macrophages are very important to the cicatrization process, since they release mediators that attract new cells like neutrophils, fibroblasts and endothelial cells to the lesion area [47]. In addition, our results showed there was also an increase in the overall amount of scar tissue cells, probably there was a high amount of mast cells. This occurred mainly when we used higher doses of Dx and in the early stages of the healing process. Mast cells are important for the inflammation phase of the repair process, as they promote the activation of macrophages and the formation of granulation tissue, cell migration and maturation of collagen fibers [48]. Based on this, we believe that Dx can stimulate the process of cell migration and speed up the closure of the wound, and consequently decrease the risk of infection. On the other hand, we observed that Dx estimulate an increase in the proportion of type I collagen, associated with a low proportion of type III collagen after 21 days of treatment. These characteristics are very important, because a high proportion of type I collagen fibers gives the tissue more resistant and force [49]. These collagen fibers exhibit covalent bonds and are more common in intact skin [50]. In general, the start of the cicatrization process produces a high deposition of type III collagen, but as the process progresses, the replacement of type III collagen by type I collagen fibers occurs [12], allowing the tissue to be more resistant to traction. In relation to elastic fibers, after treatment with Dx there was an increase in this fiber in the extracellular matrix, especially in the final phase of tissue remodeling. The role of Dx on elastic fibers was analyzed by Chung et al. (2008) [51], in the Marfan Syndrome, in which Dx preserved the elastic fibers in the thoracic aorta, probably by inhibiting the action of metalloproteinases. Thus, our findings show the induction of the proliferative capacity of cells and consequently synthesis of extracellular matrix after the application of Dx.

Cellularity and neovascularization are essential to the process of wound healing, especially in the inflammatory phase [6]. A good vascularization can provide oxygen and nutrients for the tissue which are important for the recovery of injured tissue [52]. In addition, the formation of new vessels is directly linked to the formation of a new matrix, called granulation tissue, rich in vessels and cells. When the process advances, a decrease in the

number of vessels and cells occurs, but the number of fibers increases, characterizing the mature scar tissue [6,53]. Therefore, in a new treatment is desirable to promote high levels of vessels at the start of the repair process and decreasing of the levels of vessels at the end of the process. Our findings indicate that Dx promoted high levels of new vessels at the start of the wound process and consequently high levels of cells. On the other hand, there was a reduction in vessels and cells in more advanced phases during the wound repair. These results showed that Dx was effective for ensuring a suitable environment for cell metabolic activities and providing a better skin recovery.

The excess of free radicals is usually associated with an increased inflammatory phase during the tissue repair process. In skin lesions, macrophages produce free radicals in tissue under repair, in a process known as respiratory burst [54,55]. Free radicals, reactive species of oxygen (ROS) and reactive species of Nitrogen (RNS) promote cellular oxidative stress, damaging membranes, proteins and genetic material [56,57]. In respect to ROS, it is important to consider the presence of peroxide hydrogen (H_2O_2), known as an important marker of redox metabolism and more commonly found during the inflammatory phase of the healing process [58]. In our study, as expected the levels of H_2O_2 increased on the 7th day in the groups treated with Dx, corresponding to inflammatory phase, but decreased in the later phases on days 14 and 21 in group Dx_2. In this case, the excess of H_2O_2 probably occurred in the inflammatory phase due to cell migration, of neutrophils and macrophages, that release mediators and reactive oxygen species during phagocytosis [59]. In addition, in this phase, we believe that the activation of nicotinamide adenine dinucleotide phosphate (NADPH) takes place, responsible for the generation of H_2O_2 and the activation of cell proliferation and apoptosis [60]. In our study, we suggest that an increase in the number of macrophages could justify the increase of H_2O_2 in the early repair process. On the other hand, H_2O_2 and nitric oxide (NO) showed decreased levels during the day 14 indicating that Dx was efficient to inhibit the lipid peroxidation and protein oxidation in later phase of the process. NO is an important marker for the process of cutaneous repair, as the lack of NO delays the macrophages invasion and the expression of fibrinogenic components, which can slow down the wound healing process [61]. However, the excess of NO may cause irreversible damage in the cells, impaired homeostasis and can activate various signaling cascades, such as mitogen-activated protein (MAP) or c-Jun N terminal kinase (JNK), promoting degeneration and cell death [62]. In general, it's difficult to measure NO due to the short half-life in tissues. This, nitrite (NO2) and nitrate (NO3) are the markers more commonly used [63,64]. As a result of this, in our study we measured the content of nitrite and nitrate in the scar tissue of Wistar rats.

Markers of oxidative stress are important tools in evaluating the redox balance of the healthy and damaged tissues [65]. MDA is a compound of three carbons synthesized from the peroxidation of polyunsaturated fatty acids and is generally formed during the oxidation of the cell membrane lipids [66]. During the cutaneous repair, an increase of MDA commonly occurs, mainly in the inflammatory phase, indicating damage in tissue and slowing down wound closure [67]. In our study, we observed that there was a reduction in the levels of MDA on day 7, after treatment with Dx1 and Dx2, indicating that Doxycycline can help to positively modulate the redox status of tissue in recovery. Similar results were found by Nogueira et al., 2011 after the induction of convulsive lesions following the application of pilocarpine, a parasympathomimetic alkaloid extracted from jaborandi leaves. These authors showed that Dx promoted a reduction of the lipid peroxidation in the brain, protecting the tissue from the action of free radicals. Another important marker of the action of ROS and RNS in the tissue is the high content of carbonylated protein, which indicates the protein oxidation [68,53]. In the present study, after treatment with Dx, only in the lower dose, reduced levels of protein carbonylation occurred, suggesting the antioxidant secondary action of this drug. Serra et al. (2015), evaluated the antioxidant action of doxycycline. This drug, as well as to other tetracycline, is similar to vitamin E, mainly due to the presence of a phenolic ring with multiple substitutions. This phenolic ring reacts with the free radical and generates a phenolic radical, which is relatively stable and not reactive to cellular components [69]. In this sense, in addition to acting as antimicrobial, Dx also has antioxidant secondary action protecting the tissue in recovery.

To maintain redox homeostasis in an injured tissue the action of the anti-oxidant defense system is necessary, which exhibits components such as superoxide dismutase (SOD), catalase (CAT) and glutathione S- transferase (GST) [70, 71]. SOD is responsible for the removal of superoxide radical, highly dangerous and destructive for the cells [72]. CAT enzyme is responsible for accelerating the passage of electron and decreasing the residence of H_2O_2 inside of the cells and consequently avoiding the harmful effect of this compound in the tissues [73]. This means that for a drug to be considered effective to treat lesions caused by free radicals, it is highly desirable that it stimulates the transcription and translation of these antioxidant enzymes. Our findings showed that Dx increases the activity of SOD and CAT in the tissue. The increase of CAT occurred mainly in the inflammatory phase, which corroborates with our findings in relation to levels of H_2O_2. We believed that the increase of SOD and CAT promoted the decrease of the superoxide ion ($O2^-$) and H_2O_2, protecting the tissue.

The imbalance between collagen synthesis and degradation is a common feature in cutaneous lesions, mainly in infected wounds [74]. A common consequence of this imbalance is the formation of hypertrofic scars and keloid, resulting in fibrosis and loss of tissue function [8]. The MMPs are enzymes found in acute or chronic skin wounds, which were responsible for the regulation of the deposition of collagen and degradation of the extracellular matrix, which is essential for reepithelization of the wound [16]. The excess of these enzymes may cause a disorganization of the epithelium, changes is cell-cell contact, and increase apoptosis of fibroblasts and keratinocytes [75]. In our study, we observed a decreased in the levels of MMP-10, after treatment with Dx, allowing the weaving to repair in an effective way. Other studies show that administration of Dx speeds up the closure of cutaneous wounds by inhibition of MMP-9 [76] and accelerates the recovery of gastric wounds by inhibition of MMP-2 and H_2O_2 [77]. The balance in the synthesis this enzyme is required for efficient cutaneous repair and can facilitate the cellular migration accelerating the recovery of the tissue.

In conclusion, our findings indicated that the two doses tested of the Dx can accelerate the closure the skin repair wound in Wistar rats. Dx promoted increase in the cell proliferation and migration in vivo, such as mast cells and probably fibroblasts, and macrophages in vitro. In addition, Dx was effective in the modulation of the inflammatory phase, the MMPs levels and in the reduction of oxidative species generated by wound induction. As chronic inflammation is commonly associated with oxidative stress, through our results, we suggest that Dx can modulate the skin repair process and can be used as a tool to better understand the processes involved in the repair. In addition, the effect of Dx is not dose dependent.

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4 CONCLUSÕES GERAIS

O uso de antibióticos, em especial da doxiciclina, no tratamento de feridas cutâneas não infectadas demonstrou modular o processo inflamatório com consequente progressão para fase proliferativa da cicatrização, além da maior síntese dos componentes da matriz extracelular e reepitelização, resultando no fechamento acelerado da ferida.

Por meio da revisão sistemática, ficou evidente a fragilidade dos estudos existentes na literatura sobre tratamentos antibióticos na cicatrização de feridas cutâneas não infectadas em modelos animais, uma vez que a maioria dos artigos incluídos na revisão sistemática apresentou alto risco de viés. Além disso, devido ao baixo número de estudos encontrados após a busca na literatura e por mostrar efeito no reparo do tecido cutâneo, o uso de antibióticos na cicatrização de feridas cutâneas não infectadas é um campo que possibilita maior exploração.