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LUCAS GABRIEL DE MEDEIROS DA SILVA

EVALUATION OF CHITOSAN-BASED DRESSINGS FOR CUTANEOUS WOUNDS: A REVIEW

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Trabalho de Conclusão de Curso apresentado ao curso de graduação em Farmácia da Universidade Federal do Rio Grande do Norte, como requisito parcial à obtenção do título de Bacharel em Farmácia.

Orientador: Prof. Dr. Ádley Antonini Neves de Lima
Coorientadora: Dra. Verônica da Silva Oliveira

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“No meio da dificuldade encontra-se a oportunidade”

Albert Einstein

RESUMO

Os curativos biopoliméricos estão sendo cada vez mais utilizados como uma alternativa eficaz para a cicatrização de feridas, uma vez que podem apresentar propriedades multifuncionais como: biocompatibilidade, baixa toxicidade, flexibilidade, permeabilidade, fácil remoção e dentre outras. Embora existam diversos biopolímeros para fins de cicatrização de feridas, um dos que mais se destacam, é a quitosana, devido a suas características e potencial de atuação, tal como anti-inflamatório e antimicrobiano. Neste contexto, este artigo teve como objetivo investigar estudos *in vitro* e *in vivo* de curativos de quitosana tanto isolada quanto em associações com outros componentes. Em estudos *in vitro*, foi possível analisar parâmetros como biocompatibilidade, atividade antimicrobiana e ensaios de liberação, confirmando resultados positivos para os curativos de quitosana associado com outros ativos. Nos estudos *in vivo*, avaliou-se o potencial da cicatrização em níveis macroscópicos e histológicos. Como principais resultados, foi observado que as feridas apresentaram taxa de cicatrização totalmente integralizada entre 9 e 14 dias, reepitelização completa e formação de colágeno. Dessa forma, este trabalho revelou que é possível desenvolver curativos biopoliméricos de quitosana incorporados a outros polímeros ou insumos farmacêuticos ativos, visto que os resultados são extremamente satisfatórios, tornando uma estratégia promissora para a realização de estudos clínicos no tratamento de lesões cutâneas.

Palavras-chave: Curativos poliméricos, feridas cutâneas, quitosana, ensaios biológicos, cicatrização de feridas, técnicas de caracterização.

ABSTRACT

Biopolymeric dressings are increasingly being used as an effective alternative for wound healing, since they may present multifunctional properties such as biocompatibility, low toxicity, flexibility, permeability, easy removal and many others. Although there are several biopolymers for wound healing purposes, one of the most prominent is chitosan, known it for characteristics and potential for action, such as anti-inflammatory and antimicrobial. In this context, this article aimed to investigate *in vitro* and *in vivo* studies of chitosan dressings both isolated and in associations with other components. In *in vitro* studies, it was possible to evaluate parameters such as biocompatibility, antimicrobial activity and release assays, confirming positive results for chitosan dressings associated with other components. In *in vivo* studies, it was possible to evaluate the healing potential at macroscopic and histological levels. As main results, it was observed that the wounds showed complete healing rate between 9 and 14 days, complete re-epithelialization and collagen formation. Thus, this work revealed that it is possible to develop biopolymeric chitosan dressings incorporated to other polymers or active pharmaceutical inputs, since the results are extremely satisfactory, making it a promising strategy for clinical studies in the treatment of skin lesions.

Keywords: Polymeric dressings, skin wounds, chitosan, biological assays, wound healing, characterization techniques.

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marine drugs

MDPI

Review

Evaluation of Chitosan-based Dressings for Cutaneous Wounds: a review

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Abstract: Biopolymeric dressings are increasingly being used as an effective alternative for wound healing, since they may present multifunctional properties such as biocompatibility, low toxicity, flexibility, permeability, easy removal and many others. Although there are several biopolymers for wound healing purposes, one of the most prominent is chitosan, known it for characteristics and potential for action, such as anti-inflammatory and antimicrobial. In this context, this article aimed to investigate in vitro and in vivo studies of chitosan dressings both isolated and in associations with other components. In in vitro studies, it was possible to evaluate parameters such as biocompatibility, antimicrobial activity and release assays, confirming positive results for chitosan dressings associated with other components. In in vivo studies, it was possible to evaluate the healing potential at macroscopic and histological levels. As main results, it was observed that the wounds showed complete healing rate between 9 and 14 days, complete re-epithelialization and collagen formation. Thus, this work revealed that it is possible to develop biopolymeric chitosan dressings incorporated to other polymers or active pharmaceutical inputs, since the results are extremely satisfactory, making it a promising strategy for clinical studies in the treatment of skin lesions.

Keywords: Polymeric dressings, skin wounds, chitosan, biological assays, wound healing, characterization techniques.

1. Introduction

Skin wounds consist of injuries that damage the integrity of the skin tissue, causing trauma. These wounds can be classified as acute and chronic. The first consists of a complete and orderly healing process, within a predictable time interval, depending on the depth, size and magnitude of the wound [1–3]. However, chronic wounds take longer to heal and do not follow the order of healing stages, which may present greater risks for microbial growth at the wound site [4]. Additionally, wounds can be evaluated according to their depth, being called superficial wounds, involving only the epidermis layer, having a healing time of around 10 days. While the deep dermal wound presents scarring and re-epithelialization between 10 – 21 days. Wounds that require longer healing time (>21 days) are called full-thickness wounds, due to the fact that they cause damage to the dermis and hypodermis [4].

The wound healing process requires a series of complex and dynamic events that make it possible to repair the structural integrity of the skin [5]. After the appearance of an injury, the immune system activates various intracellular and intercellular pathways that promote homeostasis of the affected tissue [6]. The human body's normal response to injury occurs in 4 distinct stages, which include homeostasis, inflammation, proliferation and remodeling. Superficial, small, clean wounds are generally associated with a short

duration of the hemostatic and inflammatory phase, this is because there is blood clot formation, which is effective in stopping the bleeding and there are small amounts of cellular debris. However, deep, large, contaminated wounds require more time to heal because the initial phases of wound healing include more time for hemostasis, removal of cell debris and necrotic tissue before granulation tissue begins to form [7].

In this context, the development of traditional dressings has partially improved the progress in the treatment of cutaneous wounds, because they aim to control blood loss and healing takes place through a natural process. Thus, these dressings usually only have the potential to cover the wound and hinder the proliferation of bacteria, but are generally powerless against recurrent wound infections and tissue healing [8]. The market for the treatment of skin wounds urgently needs more efficient products and new technologies to deal with the healing processes, infection control and skin regeneration.

Modern dressings are increasingly valued in the field of health care and innovation due to their multifunctional properties that are extremely essential and beneficial for wound care, such as anti-inflammatory, antioxidant and healing [9]. Over time, dressings have been modified using materials to provide appropriate properties and promote optimal healing. Thus, a dressing considered ideal must have the following main characteristics: (1) low adhesion to the wound, allowing easy removal; (2) removal of exudate while ensuring a certain degree of moisture for healing; (3) protection from bacterial infection; (4) low toxicity and (5) non-allergenic [1,9].

Thus, several types of modern dressings are developed based on biopolymers or synthetic polymers for the treatment of skin lesions and can be categorized as films, hydrogels, hydrocolloids, sponges, alginates, scaffolds, mats and many others. [10]. They are usually used as a flat dressing in the form of a film, which can have different shapes, or as a free-flowing gel that can be suitable for different types of wounds [8].

Among the materials used to obtain the dressings are biopolymers, which are polysaccharides of natural origin existing in various organisms and are closely related to all types of biochemical metabolism [11]. Polysaccharides can be classified into two types: terrestrial polysaccharides and marine polysaccharides [10]. Chitin is one of the most abundant marine polysaccharides in nature after cellulose, found mainly in the exoskeletons of crustaceans such as crabs and shrimp, and its main derivative is chitosan (Figure 1).

Chitosan is composed of randomly distributed N-acetyl-*D*-glucosamine and β (1 → 4)-D-glucosamine units [12]. When it reaches a partial degree of deacetylation, it becomes soluble in acid medium, allowing its wide use for obtaining solutions and hydrogels. This solubilization occurs through the protonation of the amine groups (-NH_2) at the C-2 position of the *D*-glucosamine repeating unit [13].

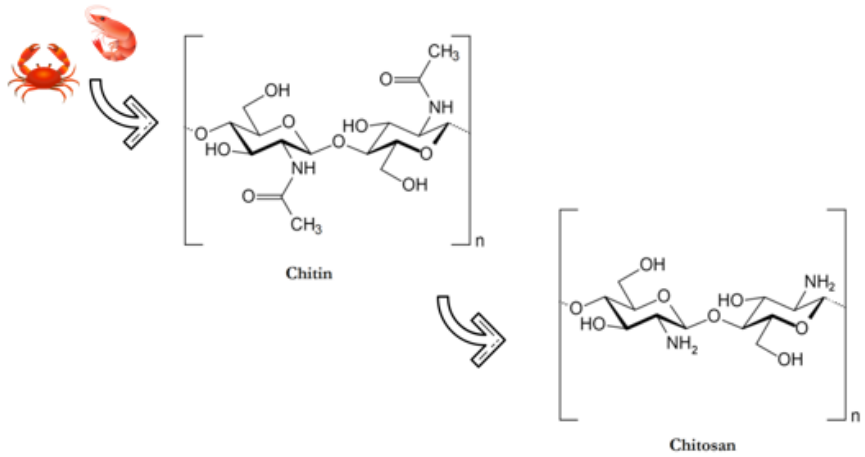

Figure 1. Structure of chitin and chitosan. Created by the author.

Chitosan has attracted significant attention from researchers due to its vast properties, such as biocompatibility, biodegradability, low toxicity and antimicrobial activity, which makes it an attractive polymeric matrix for the development of dressings [11,14–16]. Chitosan is very versatile both in terms of its applications and due to its possibilities of chemical modifications, allowing the obtaining of functionalized derivatives through the alteration of hydroxyl and amino groups [17].

An additional advantage of this polymer is the fact that it promotes the proliferation of fibroblasts [18] and prevent microbial growth in wound healing. The antibacterial property of chitosan is due to the presence of free amine groups in its structure, which can cause cellular lysis by binding to negatively charged carboxyl groups present in peptidoglycans, a constituent of the bacterial cell wall [19]. Furthermore, chitosan can induce remarkable morphological changes in fungal cells, such as structural changes and molecular disorganization, and is effective in inhibiting pathogen growth [20,21].

Therefore, this review is an approach to the different types of polymeric dressings, specifically those developed in chitosan matrix, in order to analyze and describe some of the *in vitro* (biocompatibility, antimicrobial activity and release studies) and *in vivo* (macroscopic and histopathological analysis) activities reported in the literature, since such tests become extremely essential for directing the potential application and efficacy of the dressings.

Thus, this study suggests the importance of the application of these chitosan-based dressings, as well as the tests that should be performed and investigated, so that can guarantee or direct the effectiveness of dressings as therapeutic agents for efficient wound healing. The development of new biodegradable, biocompatible dressings, capable of regulating all phases of healing, and the incorporation of biological properties, such as antimicrobial, as well as having excellent mechanical and adhesive properties to improve their performance in clinical applications, may be future scope for researchers working in this area.

2. Method

This work aimed to present a review on chitosan-based dressings, providing information described in the literature on *in vitro* and *in vivo* studies and the main characterization techniques. For this, articles were selected from databases such as Science Direct and PubMed, during the years 2015 to 2022, with the keywords wound healing; wound dressings; polymer; polymeric film; chitosan; characterization; antibacterial activity; *in vivo.*

3. *In vitro* studies

In this section some of the in vitro tests mentioned in the literature for chitosan-based polymeric dressings will be reported. The assays evaluated are related to biocompatibility studies, antimicrobial and release assays, and the main information is summarized in Table 1.

3.1. Biocompatibility

The in vitro biocompatibility of a dressing is tested by evaluating some aspects, including cell viability and hemocompatibility. These mechanisms can reduce the rate of wound healing [22].

In a skin lesion, when blood (plasma, proteins and platelets) interacts with the surface of the dressing, it can be adsorbed and cause thrombotic events [23]. In this situation, since blood is the first tissue that comes into contact with the dressing material, the dressing must have hemocompatibility to favor healing and avoid adverse effects.

The well-being or destruction of red blood cells are indicators of hemocompatibility. Generally, the lower the hemolysis value, the better the compatibility of the dressing with blood. A value of up to 5% hemolysis is allowed for dressings to be considered hemocompatible [24]. Picone et al. evaluated the hemocompatibility of a hydrogel film and reported that the dressing did not activate coagulation or fibrinolysis, due to its partial adhesiveness and non-ionic molecular structure [22].

Additionally, cytotoxicity is a standard method for evaluating the cytocompatibility of dressings, and a biomaterial can be considered cytotoxic when cell viability is less than 70%, according to the biological parameters determined by ISO10993-5 standards [22]. In a review study on chitosan-based hydrogels and their applications for drug delivery, Hamedi et al. reported that the toxicity of chitosan was insignificant [25]. Also in this review, one of the studies reported by Ribeiro et al., where the in vitro cytotoxicity assay of hydrogels tested on dermal fibroblasts obtained from the skin of rats was carried out, showed that the hydrogel degradation by-products are not cytotoxic [26].

3.2. Antimicrobial

During the process of development and evaluation of dressings, it is essential that studies are carried out to direct and prove the antimicrobial potential of these materials. Among these, there is the antimicrobial activity, since it is an essential property that helps in the healing process of a wound and prevents infections. These tests can be performed through Antibacterial Sensitivity Tests (AST) and/or determination of the Minimum Inhibitory Concentration (MIC), to verify whether or not the dressing inhibits microbial growth. [27–29].

Researchers stated that the hydrophobic character of chitosan plays an important role in penetrating the cell wall of microorganisms [30]. In gram-positive bacteria, cell wall peptidoglycans are hydrolyzed, leading to leakage of intracellular components of the bacterium. In turn, in gram-negative bacterial species, chitosan can cause changes in the permeability of the outer envelope, causing bacterial cell death, due to the impediment of nutrient transport [31].

In this context, among the gram-positive bacteria most frequently related to wound infections are *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus cereus*. While for gram-negative strains, *Pseudomonas aeruginosa* and *Escherichia coli* are reported [29,32].

The development of dressings composed of biopolymers that have intrinsic antibacterial activity and/or the incorporation of antibacterial components to these biopolymers, such as metallic nanoparticles [33–36], antibiotics, synthetic actives or of natural origin such as oils [37], have been reported and presented relevant results, providing an increase in the therapeutic potential in patients.

A major advantage of these dressings is their dual delivery, that is, the ability to load more than one active component or drug into their matrix [38]. Thus, the biofunctionality of chitosan can be enriched by combining it with antimicrobials and/or bioactives [5], for example, in studies conducted with guanidine [39], hesperidin [28], capsaicin [33], as mentioned in Table 1.

There are several types of metal nanoparticles that have different mechanisms of action to eliminate or inhibit bacterial growth, preventing the development of a resistance. Nanoparticles of silver (Ag), copper (Cu), gold (Au), magnesium (Mg), zinc (Zn), and titanium (Ti) are examples that can assist in the treatment of lesions through antibacterial effects [40].

According to studies by Zaitun Hasibuan et al., chitosan and cellulose film with silver nanoparticles as active principle were developed and the in vitro antimicrobial activity was evaluated against the bacteria *Pseudomonas aeruginosa*, *Bacillus subtilis* and the fungus *Candida albicans*. As a result, the dressings showed a large zone of inhibition for the strains tested (>10 mm), confirming both bacterial and antifungal activity [41].

In another study, Lemraski et al. developed chitosan and polyvinyl alcohol (PVA) nanofibers associated with copper nanoparticles and reported that the antibacterial activity was effective for the tested bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus cereus*) [34].

According to some studies in the literature, the antifungal activity of chitosan has been shown to be less efficient when compared to the antibacterial activity. It has been reported that the level of fungal inhibition is highly related to the concentration of chitosan. Thus, chitosan dressings can induce marked morphological changes, such as structural alterations and molecular disorganization of fungal cells [20]. Such antifungal potential results were reported in the studies by Kraisit et al., in which a film of chitosan and fluconazole was evaluated *in vitro*, resulting in the observation of significant inhibition zones for *Candida albicans.* [42].

3.3. Release Tests

Biopolymeric dressings that have a controlled drug delivery system are shown to be extremely relevant, as they improve therapeutic efficacy, reduce toxicity and increase patient adherence to treatment by releasing the active compound at a controlled rate over a certain period of time. time. During the fabrication of a dressing, the high crystallinity and large surface areas of polymer matrices potentially influence release properties [41]. In dressings that deliver a certain amount of drug over time, the rate of drug release must be controlled to avoid under and overdose [43].

Chowdhury et al. evaluated the release profile of a chitosan film loaded with neomycin for the treatment of chronic skin wounds and concluded that there was a controlled release of the drug in a pH-dependent manner. Drug release showed an increase (24%, 76% and 90%) with decreasing pH (7.5 – 4.0), respectively. Thus, the acidic medium showed greater drug release, demonstrating a potential for regular topical application for wound healing. While sustained release at higher pH was beneficial for chronic wound healing [44].

Table 1. *In vitro* assays of polymeric dressings

Polymeric matrix	Active principle	Dressing type	*In vitro* studies	Strains tested	Outcomes	Ref.
Chitosan Collagen	-	Sponge	Antibacterial – AST	*Escherichia coli* *Staphylococcus aureus*	The dressing showed antibacterial properties, effective in inhibiting the growth of gram-positive and gram-negative bacteria on the wound surface.	[27]
Chitosan Collagen	Silver nanoparticles	Scaffold	Silver Release Test	*Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	Gradual increase of the Ag ion release rate over the 7-day period. The rate of antibacterial action was > 90% against the tested bacteria, reaching 100% at the 0.3 mg/cm^3 concentration range of active release.	[35]
Chiosan Gallic acid	Copper nanoparticles	Gel	Antibacterial – Plate Count Test	*Escherichia coli* *Staphylococcus aureus*	As the concentration of the dressing increased (0, 10, 20, 30 and 40 μg mL-^{1}), the number of bacteria decreased, revealing a strong antibacterial effect.	[45]
Chitosan Honey	Gold nanoparticles Capsaicin	Nanofiber	Antibacterial – AST	*Pasteurella multocida,* *Klebsiella hinoscleromatis* *Staphylococcus pyogenes* *Vibrio vulnificus*	The dressings showed satisfactory inhibition zones, conferring antibacterial activity against the tested strains.	[33]
Chitosan Keratin	Zinc Oxide nanoparticles	Hydrogel	Cell Viability Assay Antibacterial Activity Test	*Escherichia coli* *Staphylococcus aureus*	Cell viability improved to approximately 95% after 3 and 7 days of incubation. There was proliferation of fibroblasts, confirming the increased viability. The dressings showed satisfactory action against the bacteria tested due to the bactericidal action of zinc oxide.	[29]
Chitosan Sodium alginate Calcium alginate	Magnesium	Film	Cell Migration Assay Antibacterial - AST	MRSA MRSE	Significant increase in the migration of HDFs and HUVECs cells. The films were effective to eliminate the bacteria adhered to them with a concentration lower than 6.0 x 104 for MRSA and 3.0 x 104 CFU/ml for MRSE.	[36]
Chitosan Polyvinylpyrrolidone	Titanium dioxide	Gel	Cytocompatibily Test Antibacterial – AST Hemocompatibility Test	*Escherichia coli* *Staphylococcus aureus* *Pseudomonas aeruginosa* *Bacillus subtilis*	The study indicated that NIH3T3 and L929 cells (mouse fibroblasts and embryonic cell line, respectively) grew very well after 7 days of dressing exposure, revealing biocompatibility. The dressings showed antibacterial activity, being higher against gram-positive bacteria when compared to gram-negative bacteria.	[24]

					The dressing showed a hemolysis rate of 1.14%, within the defined limit, considering the hemocompatible material.	
Chitosan Hyaluronic acid	Gentamicin	Film	Drug Release Assay Antibacterial – AST Cytocompatibily Test	*Pseudomonas aeruginosa* *Staphylooccus aureus*	Decreased rate of gentamicin release and extension of release time. The film showed good bacteriostatic capacity, having an inhibitory effect against both strains. Cell viability suggested that the film was not only non-toxic to NIH3T3 cells, but also promoted the growth of NIH3T3 cells with the participation of gentamicin.	[46]
Chitosan Sodium alginate	Mupirocin	Film	Drug Release Assay Antibacterial – AST	*Escherichia coli* *Enterococcus hirae* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Bacillus cereus* *Klebsiella pneumoniae*	Mupirocin after being 2h in the middle of release, showed a complete release rate. Films with mupirocin showed antibacterial activity that can be used to prevent serious wound colonization or infection.	[47]
Chitosan Co-Glycolic Lactic Acid Halloysite	Minocycline	Film	Drug Release Assay Antibacterial – AST	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Slow and controlled release. The antibacterial effect was greater in Staphylococcus aureus bacteria when compared to *Pseudomonas aeruginosa*.	[48]
Chitosan Sodium alginate	Hesperidin	Hydrogel	Drug Release Assay Cell Viability Assay Antibacterial Activity Test	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Sustained release of hesperidin for 14 days The hydrogels showed cytocompatibility and proliferative effect on cell growth. Dressings containing the polymers and hesperidin significantly decreased the number of colonies of the bacteria tested.	[28]
Chitosan PVA	Polyhexamethylene guanidine hydrochloride (PHMG)	Sponge	Teste de atividade antibacteriana da solução de PHMG	*Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	All strains tested showed inhibition by PHMG with MIC of 0.1%. The number of colonies counted on Müller-Hinton agar plates was less than 5.	[39]
Chitosan	Buriti oil (*Mauritia flexuosa* L.)	Gel	Antibacterial Activity (MIC)	*Staphylococcus aureus* *Klebsiella pneumoniae*	All strains tested showed inhibition by PHMG with MIC of 0.1%. The number of colonies counted on Müller-Hinton agar plates was less than 5.	[37]

HDFs – Human Dermal Fibroblasts; HUVECs - Human Umbilical Vein Endothelial Cells; MRSA – Methacillin-resistant *Staphylococcus aureus*; MRSE -Methacillin-resistant *Staphylococcus epidermidis*; CFU – Colony Forming Unit; MIC - Minimum Inhibitory Concentration; PVA – Polyvinyl Alcohol;

4. *In vivo* Studies

Once there is satisfactory evidence of in vitro tests, one can move on to in vivo studies, which are extremely important in the development of dressings, as they identify safe levels of efficacy in wound healing, both macroscopically and histologically. The anatomical/functional similarity to humans is considered when choosing an animal model. The rodent excisional wound model makes it possible to study wound healing in terms of chronology (closure) and physiology (granulation, vasculature, formation, etc.) [38].

4.1. Wound Healing

Many researchers reported obtaining chitosan-based dressings and evaluated their applications in wound healing. In most studies, the investigation was carried out through macroscopic and histopathological analyses.

Therefore, in these studies, the healing capacity of an injury in vivo is tested in animal models, using rats, mice or rabbits. To carry out the methodology, generally, a hairless part of the animal's skin is selected, and then excisional cuts are performed, with the aid of surgical instruments, such as a scalpel or biopsy punch, to promote the formation of a wound. Figure 2 illustrates the wound induction methodology in an animal model.

The parameter determined for the specification of wound healing in relation to macroscopic analysis is called contraction rate or also known as wound healing rate, as mentioned in some studies. For this, the size of the wound is measured and photographed on the day of the wound and on alternate days or not, until the last day of wound healing [32,33,39,49,50]. The rate of wound contraction is measured according to the following formula:

$$\textit{Wound contraction (\%)} = [(\textit{initial area} - \textit{final area}) / \textit{initial area}] \times 100 \quad (1)$$

Another way to evaluate macroscopic healing is by measuring the decrease in diameter during days of skin lesion observation [51]. After the period of macroscopic observation of the wounds, histopathological analysis is performed by taking skin samples from the animals in order to determine changes in tissue structure and response.

Table 2 summarizes *in vivo* studies that contain results from tests evaluating the wound contraction rate as well as the main findings from histopathological analyses.

In several studies, it was observed that between 12th and 15th days, wound healing in animals without the influence of dressings still remained incomplete, in contrast the rate of wound healing is reduced with the presence of the dressings [45,52–54].

4.1.1. Macroscopic Analysis

Pereira et al. developed chitosan-based films loaded with the fraction of *Mansoa hirsuta*, which is a Bignoniaceae plant endemic to the Brazilian semiarid region, which represents a source of phytochemicals against inflammatory processes. Excisional skin wounds measuring 5 mm in diameter were performed in the dorsal region of each mouse model. The group treated with these films achieved 40, 62 and 100% wound contraction after 5, 7 and 10 days of treatment, respectively, indicating that the *Mansoa hirsuta* fraction improved the wound healing effect of the chitosan films due to the presence of compounds present in the fraction, such as oleanoic acid and ursolic acid (terpenoids) [55].

Labib et al. investigated the wound healing potential with the use of chitosan-based dressings with the incorporation of *Melaleuca alternifolia* and *Rosmarinus officinalis* L. essential oils separately or in combination. The excision type wound was performed, with a total thickness of 2 cm and it was observed that the mixture of essential oils in the

chitosan film allowed a wound contraction rate above 80%, and the topical application resulted in a significant increase in the wound contraction percentage 2 times higher when compared to the negative control on the 14th day [49].

Ferreira et al. conducted a study aiming to evaluate the effect of a chitosan gel associated with buriti oil (*Mauritia flexuosa* L.) as a healing agent. A circular excision of the wound was performed using a 0.6 cm diameter biopsy punch in the dorsal region of rats. The animals treated daily with the formulation showed higher rates of wound retraction from the 7th day. And on day 21 there was complete healing of the lesion. Thus, inferring that the association of chitosan with buriti oil accelerated the healing process due to the high antioxidant action [37].

In another study, Lemraski and co-workers, prepared an antimicrobial dressing based on chitosan and PVA loaded with copper nanoparticles. An excision-type wound was performed on healthy, male, albino Wistar rat models. The Chitosan/PVA/Copper dressings showed a rapid wound shrinkage rate on day 3, around 35.92%. And on day 16, the group treated with these dressings showed complete healing, while the wound contraction rate in the negative control group was still at 90% [34].

Additionally, Al-Musawi et al. developed chitosan and honey-based dressings loaded with capsaicin and gold nanoparticles. To analyze the wound healing process, the dressings were used on a sectional wound on the dorsal side of rabbits. The chitosan/honey dressings associated with capsaicin and nanoparticles performed the best among the dressings tested, with a 100 percent decrease in wound size within 10 days. The researchers also noted that the prepared dressings adhered easily to the wounds without the need for a biological adhesive [33].

Li et al. developed a chitosan and collagen dressing loaded with silver nanoparticles. In this study, deep second-degree burns were induced in rats and the dressings were replaced every 2 days. Until day 7 there was no significant difference between the groups. However, the groups treated with chitosan/collagen and chitosan/collagen/silver showed a higher healing rate when compared to the natural healing group (saline) between days 10 and 14. These two groups, at day 21 showed healing rates greater than 90% [35].

In a recent study, Yue et al. prepared and evaluated an antibacterial dressing based on chitosan-PVA and polyhexamethylene guanidine hydrochloride to accelerate wound healing in infectious skin repair. A full-thickness wound model of 10 mm diameter was performed on the dorsal region of rats. After surgery, the sponges were changed every 3 days. On day 14, the wounds treated with this dressing had a wound healing rate of more than 80%, faster than the groups treated with the other dressings tested [39].

Table 2. Evalutation of healing rate and histopathological analysis of *in vivo* studies

Polymeric matrix	Active principle	Dressing type	*In vivo* studies	Method	Oberservation period	Animal model	Results	Ref.
Chitosan Poly (lactic acid)	-	Film	Wound contraction rate	Excisional wound	15 days	Rat	The animals treated with the films showed a contraction rate greater than 95% in 10 days.	[54]
			Histopathological analisys	Hematoxylin and Eosin (H&E) and Masson's trichome staining	After the 15th day		The dressings allowed more blood vessels and hair follicles to form.	
Chitosan Co-glycolic lactic acid Halloysite	Minocycline	Film	Wound contraction rate	Burn wound	12 days	Rat	Wound size reduction by 70% after 12 days without infection.	[48]
Chitosan	Chloramine	Film	Wound contraction rate	Excisional wound	10 days	Mouse	The wound healing rate reached 90% in 10 days.	[56]
			Histopathological analisys	H&E and Masson's trichrome staining	1st and 10th day		On the 1st day, tissue with a large amount of fibroblasts, while on the 10th day, the wound treated with the film showed a reorganized epithelial layer with evident stratification.	
Chitosan Sodium alginate	Pirfenidone	Film	Histopathological analisys	Excisional wound	12 days	Mouse	After 9 days, wound contraction was faster when compared to the other groups (>90%).	[53]
Chitosan	*Mansoa hirsuta*	Film	Wound contraction rate	Excisional wound	14 days	Rat	The wound contraction rate using the films was 40, 62 and 100% after 5, 7 and 10 days of treatment, respectively.	[55]
			Histopathological analisys	H&E and Masson's trichrome staining	2nd, 7th and 14th dia		The wounds treated with the dressing on the 7th day showed advanced healing and re-epithelialization with numerous vascular sprouts and keratin formation. On the 14th day, the wound presented a completely re-epithelialized area.	
Chitosan	*Melaleuca alternifolia Rosmarinus officinalis L.*	Film	Wound contraction rate	Excisional wound	14 days	Rat	The wound contraction rate was greater than 80%, when compared to the negative control, which was approximately 40%.	[49]
			Histopathological analisys	H&E staining	7th day		The samples demonstrated complete re-epithelialization on the 7th day occupied by highly cellular granulation tissue, inflammatory cells and neovessels.	

Chitosan Alginate	Hesperidin	Hydrogel	Wound contraction rate	Excisional wound	14 days	Rat	The dressings showed a healing rate of 82% and 98%, on the 7th and 14th days, respectively.	[28]
			Histopathological analisys	H&E and Masson's trichrome staining	14th day		The hydrogel showed wound contraction with epidermal formation and remodeling, in addition to better collagen deposition synthesis.	
Chitosan PVA	Silver nanoparticles	Hydrogel	Wound contraction rate	Excisional wound	12 days	Mouse	Between days 9 and 12, the animals treated with the hydrogels had a contraction rate of 99%.	[52]
			Histopathological analisys	H&E staining	12th day		On the 12th day, there was complete epidermal coverage over the surface of the wound, in addition to presenting a granulation tissue and infiltrate of inflammatory cells.	
Chitosan Oxidized dextran	-	Hidrogel	Wound contraction rate	Excisional wound	15 days	Rat	On day 10, the hydrogel wound contraction rate was 95%, on day 15, the hydrogel wounds were almost healed.	[57]
			Histopathological analisys	H&E and Masson's trichrome staining	5th, 10th e 15th day		In the 10th day, the wounds showed complete re-epithelialization, forming thicker, more organized granulation tissue, and collagen production was increased.	
Chitosan	Vitexin	Gel	Evaluation of wound diameter	Excisional wound	21 days	Rat	In the day 21, the wound diameter had decreased to approximately 2 mm.	[51]
			Histopathological analisys	H&E staining			The chitosan and vitexin gel provided re-epithelialization and wound healing in a shorter time.	
Chitosan Polyvinylpyrrolidone	Titanium dioxide	Gel	Healing rate	Excisional wound	16 days	Rat	The healing rate on the 16th day was 99.09%.	[24]
Chitosan	*Lactobacillus fermentum Lactobacillus reuteri*	Nanogel	Wound contraction rate	Excisional wound	14 days	Rat	The wounds treated with the dressings showed a contraction rate of approximately 85% on the 8th day and on the 10th day healing had been completed.	[58]
			Histopathological analisys	H&E staining	14th day		Epithelialization results were complete on day 14.	
Chitosan PVA	Coper nanoparticles	Nanofiber	Healing rate	Excisional wound	16 days	Rat	The group treated with Chitosan/PVA/Copper Nanoparticles	[34]

							dressings showed complete healing on the 16th day.	
Chitosan Honey	Capsaicin Gold nanoparticles	Nanofiber	Wound contraction rate	Excisional wound	14 days	Rabbit	The wound reached 100% contraction in 10 days.	[33]
Chitosan Collagen	Silves nanoparticles	Scaffold	Wound contraction rate	2nd degree burn wound	21 days	Rat	The chitosan/collagen and chitosan/collagen/silver nanoparticles dressings had a rate > 90% (21st day), while the saline group had a rate of 60%.	[35]
			Histopathological analisys	H&E staining	21st day		On the 7th day there was infiltration of inflammatory cells and formation of granulation tissue, as well as granulation at the edge of the wound.	
Chitosan Polyethylene oxide	Genipin extract	Mat	Healing rate	Excisional wound	14 days	Rat	The dressings showed a healing capacity of 94% after 14 days.	[59]
			Histopathological analisys	H&E staining	3th, 7th and 14th day		On the 7th day there was deposition of collagen fibers in the dermis and re-epithelialization.	

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Bektas et al. investigated the healing effect of a chitosan-based gel with vitexin, a 10 - 12 mm circular wound excision was performed in the dorsal interscapular region of each rat and wound assessment was conducted for 21 days. Progressive healing was noted with the chitosan gel associated with vitexin, where by day 21, the wound diameter had decreased by approximately 2 mm [51].

Ashoori et al. developed a chitosan-based nanogel containing a probiotic supernatant complex for application to the skin to promote wound healing. Wound excision was performed in rats and the topical formulation was administered on the wounds every day. The wounds treated with the chitosan dressing loaded with *Lactobacillus fermentum* or *Lactobacillus reuteri* resulted a completed healing process on day 10, while only the chitosan dressing showed complete healing on day 14 [58].

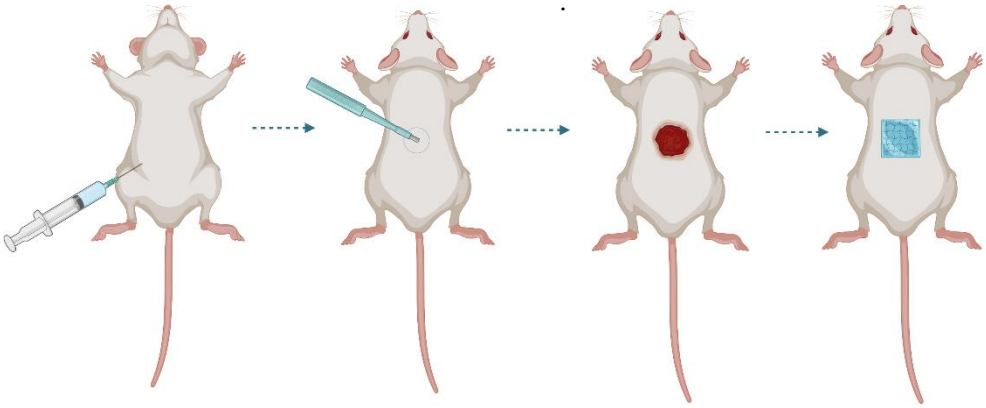

Figure 2. Methodology of wound induction in an animal model. Created with BioRender.com

Wang et al. evaluated the potential of a dressing comprising quaternized chitosan (hydroxypropyltrimethylammonium chloride chitosan), magnesium and sodium alginate for the treatment of diabetic wounds in rat models, in which the films were changed every two days. These membranes significantly promoted diabetic wound healing on day 14, with a 85% healing rate, whereas in the control group, the wounds were still large and with yellow exudate present, indicating wound infection [36].

Thangavel et al. developed a chitosan hydrogel loaded with L-Glutamic acid to treat diabetic wounds in rats. Wound excision was performed on the dorsal region of the animal and the dressing was changed every four days until complete healing. In this study, the wounds treated with the hydrogel achieved a wound shrinkage percentage of 97% in 16 days [60].

Zhai et al. obtained a hydrogel dressing, composed of chitosan and keratin loaded with zinc oxide nanoparticles to evaluate antibacterial and healing activity. The wound healing rate after the 7th and 14th day reached approximately 95%. The presence of zinc oxide nanoparticles was effective in the healing speed [29].

Bagher et al. performed a study to evaluate the effect of a hydrogel based on chitosan and alginate loaded with hesperidin for the treatment of wound healing in a mouse model and observed that dressings containing these polymers together with hesperidin had a healing rate of 82 % and 98% on the 7th and 14th day, respectively [28].

In addition, Yoon et al. prepared a photopolymerizable glycolchitosan-based hydrogel containing an inclusion complex formed between β-cyclodextrin and curcumin to evaluate the effect of accelerating wound healing. The in vivo healing test was performed using a mouse model, and the effectiveness was confirmed by measuring the remaining

area of the wound. These dressings exhibited rapidly accelerating effects on healing, and within 15 days the wound contraction rate had reduced by more than 98%. [50].

4.1.2. Histopathological Analysis

Histopathological studies are intended to provide detailed histological data on the state of a wound. To perform these tests, the tissues removed from the lesion site are fixed in 10% buffered formaldehyde aqueous solution and embedded in paraffin to perform the standard H&E and Masson's trichrome stain, which can demonstrate collagen remodeling and maturation [61].

After conducting a methodology for tissue removal and treatment, studies in the literature report the main results regarding epidermal or dermal remodeling, re-epithelialization, fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neovascularization and collagen deposition in the dermis [62], which may indicate the wound healing process. Such studies are reported in Table 2.

According to Li et al. reports, for the dressings obtained with chitosan/collagen/nanoparticle, they observed that on the first day, in each group, there was necrosis of the epidermal and dermal tissue, as well as the presence of a small quantity of inflammatory cells. On day 7 there was an infiltrate of inflammatory cells and granulation tissue formation in the groups treated with chitosan/collagen and chitosan/collagen/nanoparticle dressings. On day 14, the results for these two groups indicated epithelialization with a clear tissue structure, while in the animals treated with saline alone there was still the process of inflammatory responses and excessive granulation, without evident epithelialization [35].

In the studies by Yue et al. the chitosan-PVA and Polyhexamethylene guanidine dressing were able to significantly reduce wound inflammation. After absorbing wound exudate, the moist environment provided by the sponge was also more conducive to wound skin formation without epithelial extraction and destruction of granulation tissue during dressing changes, thereby increasing the speed of epithelialization and promoting healing. On day 14, the wounds showed basic repair and reconstruction of the skin [39].

In the histological analysis conducted by Zhang et. al, the chitosan-collagen dressings showed proliferation of fibroblasts as well as inflammatory cells and neovessels on day 3. However, on day 7, the number of new capillaries and fibroblasts increased more rapidly and orderly in the chitosan-collagen sponges. And on the 14th day of healing, there was a greater proliferation of neovessels and fibroblasts. In addition, epithelialization was observed in the boundary area around the wounds [27].

In turn, the studies of chitosan films in combination with essential oils, demonstrated on day 7, complete re-epithelialization with a large underlying area of the dermal layer, occupied by highly cellular granulation tissue together with an infiltrate of inflammatory cells and neovessels. By day 14, the wound healing process was advanced with complete re-epithelialization of the epidermal area. In addition, the dermal layer showed less granulation tissue area rich in fibroblasts [49].

Ferreira et al. observed that in the initial days of injury, the animals treated with chitosan gel associated with buriti oil showed the presence of fibrin and fewer neutrophils concentrated in the wound region and more diffuse in the dermis. However, we observed the presence of macrophages infiltrate with light intensity in the dermis, besides some eosinophils and neoformed capillaries, indicating that the granulation tissue progressively invades the space of the incision. On day 14, it was reported the decrease of inflammation with the gradual increase of collagen deposition in the incisional scar, besides the observation of some macrophages and fibroblasts. Still on day 14, complete re-epithelialization was observed in the epidermis, and on day 21 the animals were completely healed, with total re-epithelialization [37].

Thangavel et al. reported in their study that dermal reconstruction could be assessed by proliferation, remodeling and maturation at the site of injury. Diabetic wounds treated with chitosan hydrogel and L-glutamic acid showed an increase in fibroblasts, collagen synthesis and deposition [60].

In the study by Pereira et al. it was possible to see that the wound treated with chitosan films and *Mansoa hirsuta* fraction presented, on day 2, an area of ulceration with the presence of crust, purulent fibrin exudate and inflammatory infiltrate in the underlying connective tissue area. On day 7, these wounds showed a characteristic area of advanced healing and re-epithelialization, and the presence of numerous vascular sprouts with few cell layers, as well as the formation of a thin keratin layer. After 14 days, the group treated with this film showed a completely re-epithelialized area [55].

Bagher et al. also decided to deepen their studies through histopathological analysis, and with this they noted that the groups treated with chitosan and alginate hydrogels loaded with hesperidin exhibited granulation tissue formation and epidermal proliferation, as well as remodeling. Several mechanisms have been suggested to explain the effect of hesperidin on wound healing, such as eliminating free radicals, suppressing the activation of proinflammatory cytokines such as IL-1β, IL-8 and TNF-α, increasing the capacity of fibroblasts, and endothelial cell division which are essential for the regeneration of injured tissues [28].

5. Dressing Characterization Techniques

One of the relevant ways that researchers use to assess the safety and efficacy of a dressing in relation to its characteristics and physicochemical properties is the use of physicochemical and mechanical characterization techniques. However, to date, there is no official standard to characterize dressings. Characterization consists of methods related to the use and applicability of these products, such as physical, microscopic appearance, identification of components of a sample, fluid absorption capacity, etc. [32]. Table 3 presents the main methods frequently reported for the physicochemical characterization of dressings.

5.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) magnifies the image from 10 to 500,000 times enabling the analysis and characterization of the size and shape of materials at the nanoscale. A beam of electrons is emitted along a sample and the signals emitted by the sample are detected and transformed into images. At higher resolutions it is also possible to determine the atomic arrangement of crystal structures [63]. In the analysis of dressings, the SEM can be applied to films, hydrogels, sponges, membranes and other materials, to evaluate parameters such as porosity and thickness of the material and, from this analysis, improve the development of the formulation [64].

Morgado et al. produced a membrane composed of chitosan and containing ibuprofen-cyclodextrin. The SEM analysis showed that the membranes showed a higher porosity than the CS/PVA membranes developed previously. This feature is very important, as it allows the absorption of wound exudate, keeping the environment moist and aiding in the penetration of cells and the diffusion of nutrients [65].

5.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis uses the atomic vibrations of a sample subjected to infrared radiation to determine functional groups, bond types and molecular conformations about it. The peaks produced in the spectrum are specific for each type of interaction and, from them, the interpretation is made. In dressings, FTIR is applied to assess the interaction between the active ingredient and the matrix [66,67].

Kenawy et al. [68], for example, developed a membrane with healing properties using a matrix of chitosan and gelatin and incorporating cinnamaldehyde as an antibacterial

agent. The free NH_2 groups of chitosan and gelatin were bound to the free aldehyde groups of the active. These bonds were confirmed by FTIR, where a band was observed at 1431-1444 cm^{-1} (which corresponds to the interaction of the active aldehyde with NH_2 from gelatin and chitosan). The peaks at 1665 cm^{-1} did not indicate the presence of free aldehydes, showing that there was a satisfactory interaction between the polymers and the cimaldehyde.

5.3. Thermal analysis

Differential Scanning Calorimetry (DSC) and Thermogravimetry Analysis (TGA) are thermal analysis techniques used to identify a compound according to its degradation products. In TG, the mass loss as the temperature changes is evaluated. In DSC, endothermic and exothermic events that occur according to temperature variation are analyzed [69]. The techniques can be used separately or together, for better analysis of the results. In the characterization of dressings these methods can be used to evaluate the thermal stability of the preparations and their degradation products match that of the materials used, as in Moghadas et al. where the TG assay showed that at the temperature of 200 to 400 ºC there was weight loss associated with deacetylation of chitosan, which is consistent with what was observed in other studies. In addition, higher thermal stability was observed for the films obtained with the chitosan and montmorillonite biofunctionalized with chitosan sulfate chains, due to the cross-linking of the chitosan networks [70].

5.4 Swelling

Swelling capacity is a parameter related to wound infection control, because a dressing that can absorb water and wound exudates helps keep the wound environment moist, prevents airborne infections, and allows nutrients to enter. The optimal swelling rate is 100-900% and varies according to the degree of cross-linking of the matrix [71,72].

5.5 Mechanical Properties

When a dressing stretches, it should not break easily without restricting or completely nullifying the desired application. The strength and durability of a dressing are very important properties to preserve the wound from further injury, so considerable improvements in the mechanical properties of polymeric dressings are sorely needed [73]. The mechanical properties of dressing materials, such as tensile, compression, and elasticity, can be analyzed by the universal testing machine [74].

Table 3. Main techniques for physicochemical characterization and mechanical properties

Physicochemical attributes	Analysis objective	Determination/Instruments	Ref.
Surface morphology and drug distribution in dressings	A smooth structure is expected, with no voids and no disruption	SEM	[75]
Interactions between polymer and active component	Confirm the purity of the raw materials and investigate active component and polymer compatibility	FTIR	[55,75]
Thermal behavior	Evaluate the thermal stability of the prepared dressings and check the compatibility of the dressing components and the state of the drug molecules within the dressing	DSC, TGA	[75,76]
Thickness	A film-type dressing is expected to be thin, with uniform mechanical properties and	SEM; The thickness is measured by taking the image on the cross section; Digital micrometer.	[53,55,76]

	homogeneous distribution of the active ingredient		
Swelling	Water absorption capacity of the functional dressing	Weigh the dry dressing initially and then swell it, measuring the expansion film diameter	[36,76]
Mechanical properties	Tensile strength and elongation percentage at break to verify dressing integrity	Texture analyzer Universal Testing Machine (UTM) according to ASTM D882	[3,6,7]

6. Conclusion

A dressing that possesses multifunctional features of hemostasis, controlled release, antibacterial property, biocompatibility, biodegradability, and fluid absorption are desirable candidates for improving wound healing that will benefit patients in wound care.

Chitosan-based dressings can come in different forms such as hydrogels, gels, films, foams and nanofibers. The use of biopolymeric dressings associated with other bioactive agents has shown relevance in the results of *in vitro* and *in vivo* studies, due to the improvement of antibacterial and anti-inflammatory activities, biocompatibility, and sustained drug release. The addition of metallic nanoparticles, natural agents, or synthetic antibiotics in biopolymeric dressings enhances antibacterial activity, showing that the dressings can protect from bacterial infections. These properties assist in rapid wound contraction rate, tissue remodeling, granular tissue formation, and collagen deposition.

Although chitosan dressings show interesting results in reported in vitro and in vivo studies for wound treatment, few studies on dressing development have reached clinical trials. Therefore, investment in the continuation of these innovative studies and the entry of the products into the pharmaceutical market is necessary, highlighting the importance of the application of these dressings for efficient wound healing.

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Shortcuts

- **Manuscript Submission Overview**
- **Manuscript Preparation**

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