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Chitosan Hydrogel in combination with Nerolidol for healing wounds

Maria Onaira Gonçalves Ferreira[a], Layara Lorrana Ribeiro Leite[a], Idglan Sá de Lima[a], Humberto Medeiros Barreto[b], Lívio César Cunha Nunes[a], Alessandra Braga Ribeiro[c,*], Josy Anteveli Osajima[a], Edson Cavalcanti da Silva Filho[a]

[a] Graduate Program in Materials Science, Federal University of Piauí. Campus Universitário Ministro Petrônio Portella, Teresina, PI CEP 64049-550, Brazil
[b] Microbiology Research Laboratory, Federal University of Piauí. Campus Universitário Ministro Petrônio Portella, Teresina, PI CEP 64049-550, Brazil
[c] Program of Scientific and Regional Development-CNPq, Federal University of Piauí. Campus Universitário Ministro Petrônio Portella, Teresina, PI CEP 64049-550, Brazil

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ABSTRACT

Chitosan is a natural polymer with antibacterial property, that is biodegradable, extremely abundant and non-toxic. This study aimed to develop and characterize chitosan hydrogels in combination with nerolidol, in order to optimize the antimicrobial and healing properties. The hydrogels were prepared using a reaction of the chitosan with acetic acid solution, followed by the addition of 2 or 4% of the nerolidol. Using thermogravimetry, differential scanning calorimetry and infrared spectroscopy, the incorporation of nerolidol in the hydrogel was confirmed. Direct contact tests using hydrogels and *Staphylococcus aureus* showed a synergistic effect in the materials, enabling total inhibition of bacterial growth. The hydrogel containing 2% nerolidol showed excellent healing effects. The beginning of re-epithelialization and reorganization of collagen was already observed on the 7th day of treatment. The material created proofed to be promising as a healing and antibacterial agent.

1. Introduction

Chitosan is a cationic biopolymer obtained from the deacetylation of the chitin present in the exoskeletons, especially, crustaceans (crab, lobster, shrimp, etc.). This biopolymer has been widely studied for its use in several areas, as it presents biocompatibility, biodegradability, non-toxicity, mucoadhesion, antimicrobial activity, natural origin and low cost (Moreira et al., 2009). The chitosan possesses hydroxyl groups, which enable hydrogel formation through the protonation of cationic groups and consequently its swelling (Mukhopadhyaya, Mishrab, Ranac, & Kundua, 2012).

The cicatricial process of a wound involves different steps, including hemostasis, inflammation, migration, proliferation and cellular maturation (Zahedia, Rezaeiana, Ranaei-Siadat, Jafaria, & Supaphol, 2010). Faster wound healing will depend on the absence of infections. It is therefore necessary to use dressings capable of maintaining the humidity, allowing gas exchange, avoiding the entrance and proliferation of bacteria and molds, which are at the same time easy to remove (Chen et al., 2009; Kumar et al., 2012; Wang, Zhu, Xue, & Wu, 2012). In this context, the Chitosan Hydrogel (CH) is appropriated to be used as a healing dressing (Radhakumarya, Antontyb, & Sreenivasana, 2011).

Despite of this area being poorly explored commercially, nowadays the chitosan in the hydrogel form with healing purpose properties is receiving more attention nowadays. This attention can could be explained since by the fact that the chitosan is able to stimulate the fibroblast proliferation and the migration of neutrophils. It also inhibits the growth of inflammatory cells, can activate macrophages and shows antimicrobial activity against bacteria and mold. Chitosan also works in the re-epithelialization and stimulation of immune system, providing a lower degree of fibroplasia, while presenting permeability to oxygen. Chitosan is atoxic as well as bioadhesive, it shows antitumor activity and has hemostatic potential, among other properties (Azad, Sermsintham, Chandrkrachang, & Stevens, 2004; Berger et al., 2004; Minami, Okamoto, Hamada, Fukumoto, & Shigemasa, 2011; Jayakumar, Prabaharan, Kumar, Nair, & Tamura, 2011; Jin, Ling, He, & Zhang, 2007; Park et al., 2009).

In this study the substance to be combined with CH was nerolidol; an alicyclic sesquiterpene, found in essential oils from several plants, which possesses pharmacological properties such as antineoplasic, leishmanicidal, anxiolytic, larvicidal, antioxidant and

* Corresponding author.
E-mail address: alessandra.bragaribeiro@gmail.com (A.B. Ribeiro).

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antimicrobial. Besides this nerolidol acts as an enhancer of permeation of drugs in transdermal form (Arruda, D'Alexandri, Katzin, & Uliana, 2005; Costa et al., 2009; Koudou, Abena, Ngaissona, & Bessiére, 2005; Marques et al., 2010; Nogueira, Sousa, & Freitas, 2013; Pacifico et al., 2008).

The antimicrobial capacity of chitosan formulations has been thoroughly reported on. To the best of our knowledge, data related to biological application field combining CH with nerolidol are not available in the literature. Looking at the perspective of combining CH and nerolidol, it is possible to enhance the biological potential of these materials for their use in the pharmaceutical field with the goal of developing new treatments for the wound healing, preventing further complications, and in parallel, producing a dressing at low cost and with the appeal of a natural origin. In this context, the present study aimed to develop and characterize chitosan hydrogel (CH) in combination with nerolidol, and additionally, performing in vitro and in vivo evaluations of this combination in relation to its antimicrobial and healing properties, respectively.

2. Experimental section

2.1. Chemicals

Isomers cis and *trans*-nerolidol (NRL) was obtained from Sigma-Adrich (St. Louis, USA) and chitosan with a medium degree of acetylation (95%) and viscosity 405 cP was purchased from Primex. The other compounds showed at least 98% purity and were acquired as follows: acetic acid (VETEC®), sodium hydroxide (DINÂMICA®), Brain Heart Infusion and Mueller Hinton media (HIMEDIA®), Mueller Hinton with sodium chloride (IMPEX®). Ultrapure water was obtained using Milli Q® system (Millipore Corporate).

2.2. Synthesis of hydrogel

Chitosan Hydrogel (CH) was prepared using chitosan and an acetic acid solution (2%, v/v) in a ratio of mass: solvent of 2:98 (w/v) with a final pH of 3. The solution was kept under magnetic stirring for 30 min.

2.3. Chitosan Hydrogel in combination with Nerolidol (CHN)

In the preparation of CH combined with nerolidol, 1 mL of the sesquiterpene was added to 50 mL of CH and the mixture was kept under magnetic stirring for 10 min. This way CH with 2% of nerolidol (CHN2) was obtained. A similar procedure was performed to formulate CHN4, which contains 4% of nerolidol. Both formulations had their pH adjusted to 4 with a buffer solution.

2.4. Characterization of hydrogels

To evaluate the thermal stability of hydrogel, thermogravimetry (TG) and differential scanning calorimetry (DSC) were used. The thermogravimetric and calorimetric curves were obtained from SDT Q600 V20.9 Build 20, DSC-TGA Standard model, with 10 °C min^{-1} under nitrogen atmosphere, in a sample holder of alumina, with a temperature range from 0 to 327 °C, and with an approximate weight of 10 mg. Infrared spectra of pure CH or CH in combination with nerolidol were obtained by spectrometer FTIR Bomem MB Series in 32 scans, ranging from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

2.5. Antimicrobial activity

The antimicrobial test was carried out using a Gram-positive bacteria, *Staphylococcus aureus* strain ATCC 25923, kept in nutrient media at 4 °C.

2.5.1. Inoculum preparation

The *S. aureus* culture was prepared by transferring the inoculum from the nutrient media to the Brain Heart Infusion media (BHI), following incubation at 37 °C/24 h. The bacterium suspension was standardized using a 0.5 McFarland standard, which corresponds with approximately 1.5×10^8 CFU/mL (Colony Forming Unit- CFU).

2.5.2. Direct contact test

A volume of 100 μL of bacteria suspension (1.5×10^6 CFU/mL) was added using a spread plate method, containing Mueller Hinton medium. On the surface of each plate a glass slide was added with 100 μL test solution spread on top (pure nerolidol; 2% nerolidol solution; 4% nerolidol solution; CH; CHN2; CHN4); after which the plates were incubated at 37 °C/24 h.

Positive control of normal properly bacterial growth was performed, and the inhibitory potential of the solvent was simultaneously tested using acetic acid solution at pH 3, acetic acid solution at pH 4, acetic acid solution at pH 5 and acetic acid solution at pH 6. All the assays were performed in triplicate.

2.6. Wounds healing assay

In accordance with the regulations concerning animal assays (n. 11.794, October of 2008) and following the recommendations described by the National Council for the Control of Experimentation on Animals this study was approved by the Ethics Committee for Experimentation on Animals of the Federal University of Piauí under protocol number 073/14. All the chirurgical procedures were performed under anesthesia using 10% ketamine chloride and the muscle relaxant xylazine chloride aiming to minimize the pain and suffering of the animals.

2.6.1. Animals

Mice, strain swiss (*Mus musculus*), adult, albinos, male and female, between the age of 2–4 months, body weight around 25–30 g, from Central Vivarium of the Center for Agricultural Sciences – CCA of the Federal University of Piauí. The animals were kept under monitored conditions with constant temperatures (26 ± 1 °C), food and water ad libitum, and a dark/light cycle of 12 h. They were divided in groups of 9 animals, according to the different treatments as following: physiological saline (PS), Collagenase Clostridium histolyticum (CC) were used as a negative and positive control; Chistosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2) and Chitosan Hydrogel with 4% Nerolidol (CHN4) were the formulations used to treat the wounds.

2.6.2. Procedure to produce the wound

After intramuscular anesthesia using xylazine hydrochloride at a dose of 0.04 mL and ketamine hydrochloride at a dose of 0.08 mL/100 g, hair removal from the dorsal region was performed. A circular part of the skin and subcutaneous tissue with a 0.6 cm diameter were removed using an electric punch, forming a wound in the skin that exposed the dorsal fascia. After this, the animals were treated daily with the ointment/saline/chitosan hydrogel, after which they were returned to their respective cages and kept under observation.

2.6.3. Treatment of wounds

The wounds were treated and evaluated on a daily basis. The wounds were protected with a bandage, which consisted of a first layer of gauze and a second layer of crepe bandage. Treatment for each group was administered topically at the injured area, always in the same period of the day and following the protocol strictly, using the products designated for each group. The wounds were cleaned with saline (0.9%) before the daily application of the products (Rahal, Rocha, Blessa, Iwabe, & Crocci, 2001; Santos et al., 2002).

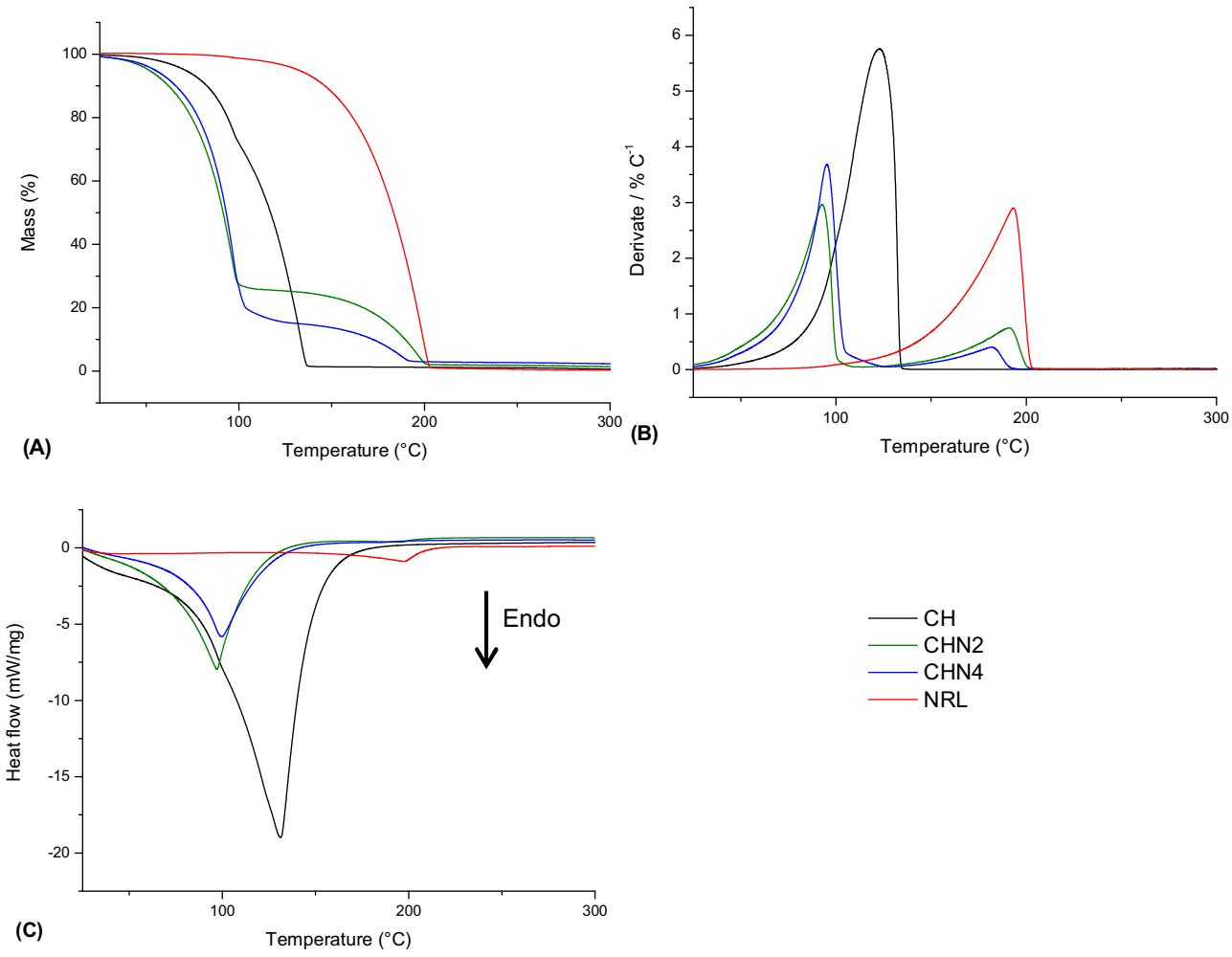

Fig. 1. TG (A), DTG (B) and DSC (C) curves for CH, nerolidol (NRL), CH with 2% Nerolidol (CHN2) and CH with 4% Nerolidol (CHN4).

Euthanasia was conducted on three animals of each group after 7, 14 and 21 days. This procedure was performed using an overdose of sodium pentobarbital with a dose of 10–15 mg/100 g of body weight, intraperitoneally, followed by excision of the skin flap for histological analysis, finishing the study after a total of 21 days of treatment.

2.6.4. Macroscopic evaluation of cutaneous lesion in mice

The animals treated with the active compounds as well as the control groups were observed daily in relation to the lesion repairment, which referred to the presence or absence of edema, exsudate and scab and the color of the wound. A photographic registration of the wound of all animals was made and the lesions were measured using an analogical pachymeter in the 1st, 7th, 14th and 21th day of treatment.

2.6.5. Histological evaluation of cutaneous lesion in mice

After 7, 14 and 21 days after the euthanasia procedure, a circle fragment of dorsal skin reaching the central and surrounding area with approximately 0.6 cm, was dissected (Rahal et al., 2001). All the samples of cutaneous lesions were fixed in 10% formalin for further histological analysis. The examination of each histological sample was performed under an optical microscope using a 100X magnitude, aiming to verify the inflammatory and healing process, observing the presence of granulation tissue, vascular proliferation, chronic and acute inflammation, as well as the presence of collagen and re-epithelization.

2.6.6. Statistical analysis

Data were analyzed using Graph Pad Prism Version 6.0 software, and the results of healing on the 1st, 7th, 14th and 21th day of treatment were expressed by the significance of the difference between the mean using variance analysis (ANOVA) with $p \leq 0{,}05$.

3. Results and discussion

3.1. Characterization of hydrogel

3.1.1. Thermal analysis

In Fig. 1, TG, DTG and DSC curves related to pure CH and those combined with different concentrations of nerolidol are shown.

Fig. 1. The nerolidol presented better stability compared the oil associated with chitosan (CHN2 and CHN4). In relation CHN2 and CHN4 was possible to verify two different thermal events, the first one was related with the decomposition of hydrogel in the temperature range of 27–102 °C and the second was related with the decomposition of nerolidol between 102 and 202 °C. The TG results can be better visualized using their derivatives (Fig. 1B), highlighting two events: the decomposition of hydrogel, followed by the decomposition of nerolidol, that can be confirmed by the DTG curve of nerolidol, which demonstrated that the decomposition event is present in the same area as the second event for the CHN2 and CHN4 materials. Considering the facts above mentioned we can deduce that there is a possible physical interaction between the chi-

Table 1
Enthalpy, initial temperature and maximum decomposition temperature for Chitosan Hydrogel (CH), Nerolidol (NRL), Chitosan Hydrogel with 2% Nerolidol (CHN2), and Chitosan Hydrogel with 4% Nerolidol (CHN4).

Material	Enthalpy (J/g)	Initial temperature of decomposition (°C)	Maximum temperature of decomposition (°C)
CH	2677.0	92.8	110.63
NRL	177.7	165.3	198.7
CHN2	2267.0	43.2	75.8
CHN4	2263.0	78.3	99.6

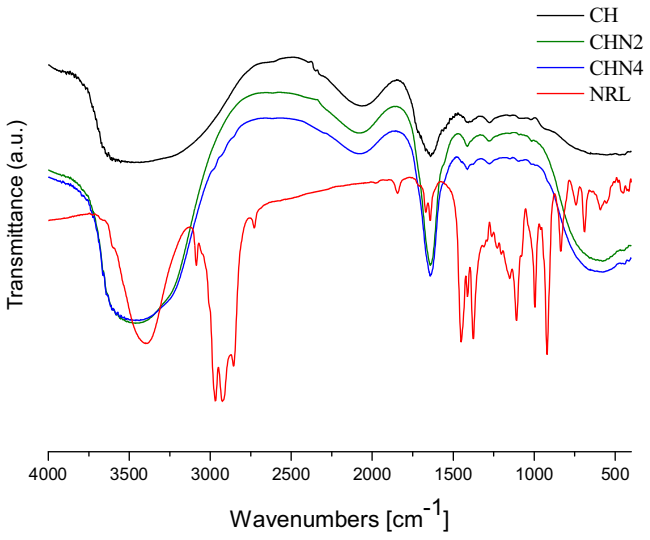

Fig. 2. FTIR profile of Chitosan Hydrogel (CH), nerolidol (NRL), Chitosan Hydrogel with 2% Nerolidol (CHN2) and Chitosan Hydrogel with 4% Nerolidol (CHN4).

tosan hydrogel and nerolidol, since the thermal events of isolated materials remain in the same range of the materials in association.

In the DSC analysis (Fig. 1C) it was possible to confirm that the thermal events observed in the TG curves are all related with a mass lost. The events for all tested materials were endothermic, which means that the heat absorption happened without losing mass. The energy necessary for the process to take place was 177.7; 2677; 2267 and 2263 J/g for NRL, CH, CHN2 and CHN4, respectively. This shows an intermediate energy for the CH combined with nerolidol when compared with pure CH and NRL, which demonstrates the incorporation of nerolidol in the hydrogels.

The fact that the nerolidol absorbed less energy to decompose itself causes a decrease in the energy used for CH combined with nerolidol in comparison to pure CH, moreover, a deslocalization temperature happened. These changes were more visible in CHN2. Table 1 sums up the enthalpy values and temperatures of events for the pure CH and the CH combined with nerolidol.

3.1.2. Infrared spectroscopy

Using the Fourier transform infrared spectroscopy (FTIR) it was possible to identify functional groups present in the structure of the material related to interactions of the molecules or atoms with electromagnetic radiation at a molecular vibration process. Fig. 2 illustrates the spectra of pure chitosan hydrogel, chitosan hydrogel combined with nerolidol (CH, CHN2 and CHN4) and nerolidol (NRL).

Fig. 2. CH showed the following band features: moderately intense band of axial deformation of NH and a stretching vibration of OH in the 3550–3100 cm^{-1} region, showing a large expansion caused by numerous interactions proving the hydrogel formation. Around 1650 cm^{-1} an axial deformation C=O appears from amide, and at 1465–1423 cm^{-1} the deformation of CH_2 and OH appears (Silverstein & Webster, 2013; Sousa, Silva Filho, & Airoldi, 2009).

In CH there is overlap between the bands related to the C—H bond of methyl groups present in chitosan. The same happened for the CHN2, however, in the CHN4 there was a presence of small shoulder in the 2900 cm^{-1} region due to the greater amount of NRL, since this substance also presents absorption in this region. In the 3100–3550 cm^{-1} region there was a broadening and an increase of intensity in hydrogels combined with nerolidol, as compared to the NRL, which was relative to OH stretching vibrations verified in both materials, indicating the existence of different interactions in gel formation in the presence of NRL (Boributh, Chanachai, & Jiraratananon, 2009; Silverstein & Webster, 2013).

The band 1600 cm^{-1} showed the intensity increased in both bands of CHN2 and CHN4, although in CHN4 the enlargement was smaller. The 1500–1700 cm^{-1} region is highlighted by the presence of bands of deformations as the C=O axial and N—H angular from chitosan, besides the deformation of the alkenes present in nerolidol, it happened the incorporation of characteristic groups from NRL and CH in combined hydrogels, causing the increased intensity and signal of flare. In the 1400 cm^{-1} region occurred an overlap of alkanes bands of the groups CH_3 and CH_2 present in the NRL (Silverstein & Webster, 2013).

3.2. Antibacterial activity

3.2.1. Direct contact test

S. aureus was the micro-organism chosen for these tests, since it is the main cause of many infections in the human body. Despite being commonly found in the skin, this micro-organism is able to penetrate into the body. When the immune system is depressed it can cause diseases. It is therefore considered an opportunistic bacterium. Additionally, its resistance to several drugs has been a challenge for the pharmaceutical industry, and therefore, the ways of overcoming these resistance mechanisms have been frequently studied (Ratti & Sousa, 2009).

The direct contact test using control solutions as saline and acetic acid at different pH (3–6) was performed to ensure that the antibacterial activity of hydrogel is not influenced by acid solution. Our results demonstrated that acetic acid solution (pH 3–6) has no inhibitory effect on the growth *S. aureus* (Please see "Supplementary material").

Fig. 3 demonstrates the antibacterial action of nerolidol when applied by itself or in combination with chitosan hydrogel. Nerolidol is a hydrophobic compound and has an affinity with the cytoplasmic membrane, when it is spread on the wall. The compound interacts with the phospholipids altering the permeability and causing the loss of important material for the bacteria, as the ion K^+, which is considered a typical behaviour of terpenic alcohol and it could explain the inhibition on the growth and proliferation of the bacteria *S. aureus* (Inoue et al., 2004; Park et al., 2009). In Fig. 3B, using the direct contact test, is it possible to verify the efficiency of the sesquiterpene for the total inhibition of the growth of colonies.

Fig. 3. The synergism among properties of chitosan hydrogel and nerolidol is clear and can be observed in Fig. 3C and D. The nerolidol in concentrations of 2 and 4% was not able to completely inhibit the bacterial growth. On the other hand, the complete inhibition (100%)

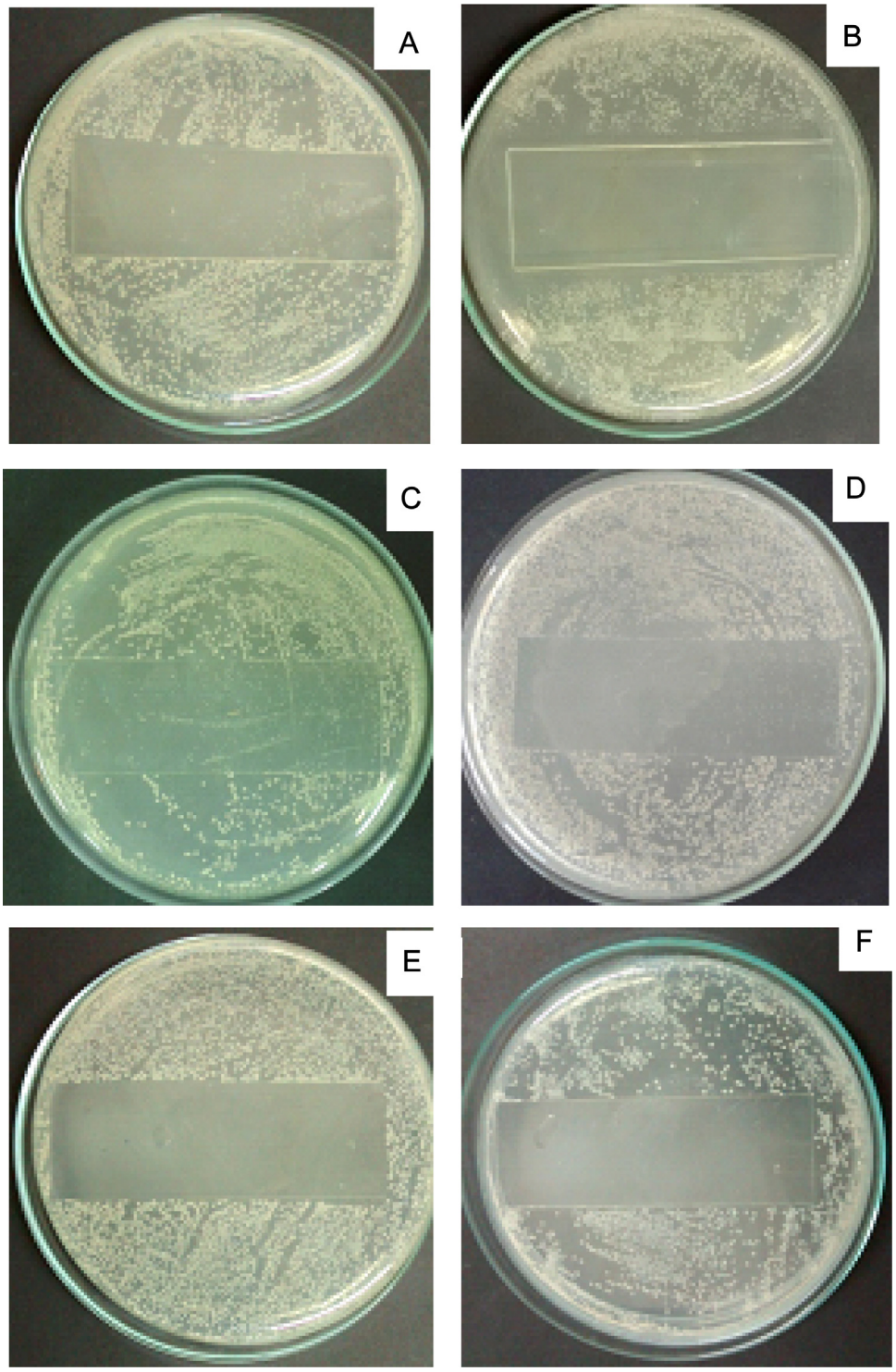

Fig. 3. Contact direct test using CH (A), NRL (B), NRL 2% (C), NRL 4% (D), CHN2 (E) and CHN4 (F) and *S. aureus* standard (ATCC 25923).

was observed when the NRL in both concentrations (2 and 4%) were combined with CH. The long hydrocarbon chain, the alcohols groups, and the hydrophobic character of nerolidol are also related to its antibacterial activity, since these properties allow a greater interaction with the membrane, increasing their permeability to other antibacterial agents, additionally, the antimicrobial activity is dependent of the number of carbons in the nerolidol hydrophobic chain and hydroxyl groups (Brehm-Stecher & Johnson, 2003; Inoue et al., 2004). This explains the results obtained by the direct contact test using CH, CHN2 and CHN4. Although CH partially inhibits the growth of *S. aureus*, the combination of CH with nerolidol (CHN2 and CHN4) provided the complete inhibition of *S. aureus* growth. In this case, only 2% of nerolidol associated with the chitosan hydrogel was enough to totally inhibit the bacterial growth in the area of direct contact with *S. aureus*. Therefore, there was an increase in the action of chitosan hydrogel, probably due to the capacity of NRL to enhance the plasma membrane permeability of the bacterium, thereby allowing greater action of chitosan, generating a combined effect of the substances (Brehm-Stecher & Johnson, 2003).

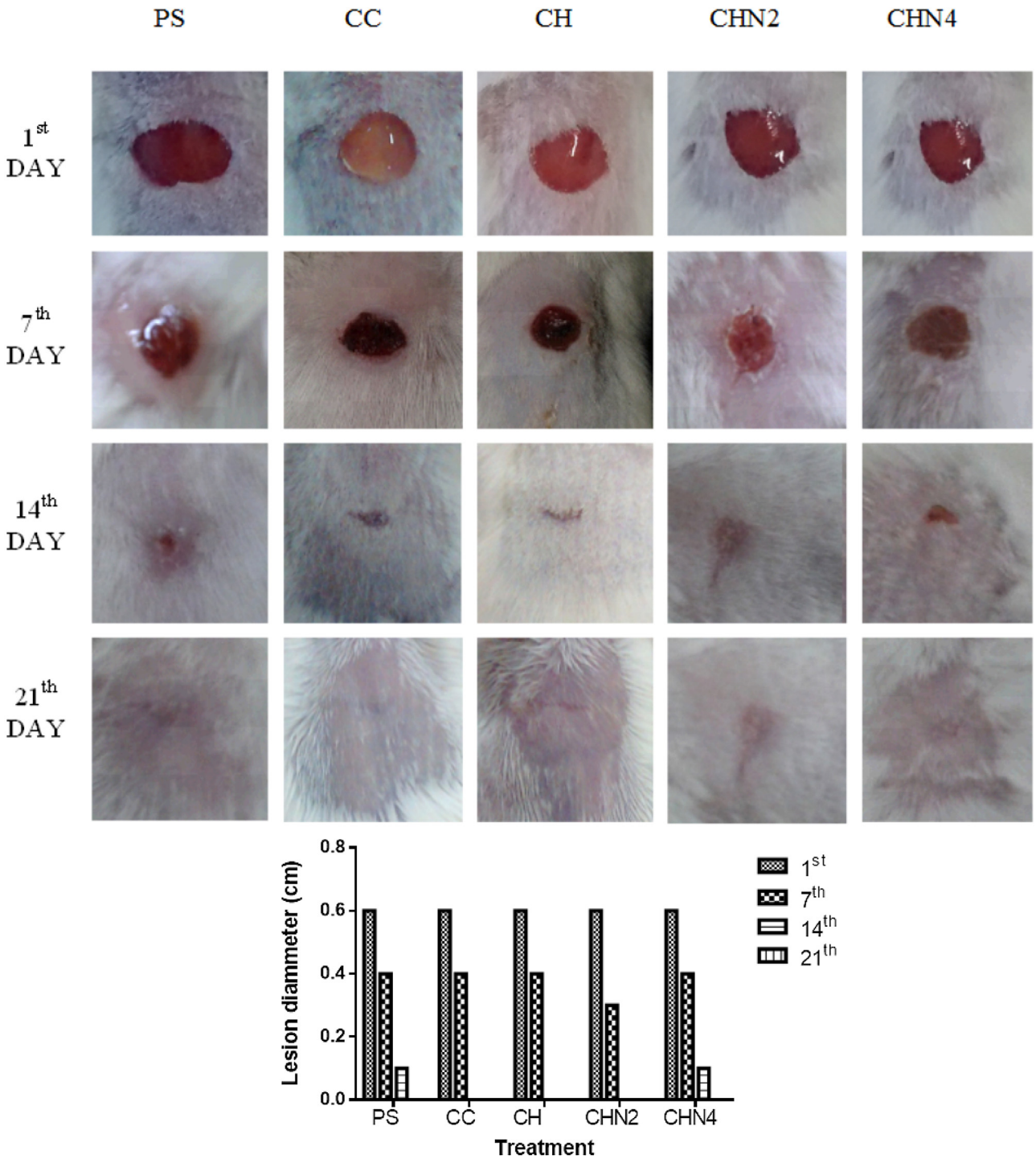

Fig. 4. Macroscopic analysis of cutaneous wounds in the days 1, 7, 14 e 21 of treatment with Physiological Saline (PS), Collagenase *Clostridium histolyticum* (CC), Chitosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2), Chitosan Hydrogel with 4% Nerolidol (CHN4). Bar graph showing the diameter of the wound in mice on the 1st, 7th, 14th e 21st day of treatment using Physiological Saline (PS), Collagenase *Clostridium histolyticum* (CC), Chistosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2), Chitosan Hydrogel with 4% Nerolidol (CHN4).

The chitosan presented as hydrogel, possesses protonated cationic groups and this may increase the electrostatic interaction of chitosan with the bacteria cell wall. The cell wall of Gram-positive bacteria is essentially formed by teichoic acid linked to the peptidoglycan, and this acid presents phosphate groups which charge the wall negatively. The interaction between the chitosan and phosphate groups destabilize the bacterial wall and this can interfere with osmosis process and biosynthesis (Alvarenga, Schwan, Schwan-Estrada, & Bravo-Martins, 2007; Carrick et al., 2010).

With this result it is possible to verify that a smaller quantity of NRL is required to completely inhibit the growth of *S. aureus* when the oil is in association with CH, since the association of this materials promotes a synergistic antimicrobial effect. The NRL in its turn enhances the antibacterial action of CH, thus there is a synergistic effect of the substances and this improves the range of applications of these combined materials. Besides this, there are benefits of lower costs, greater bioavailability and properly antimicrobial activity (Miguel, Ribeiro, Brancal, Coutinho, & Correia, 2014).

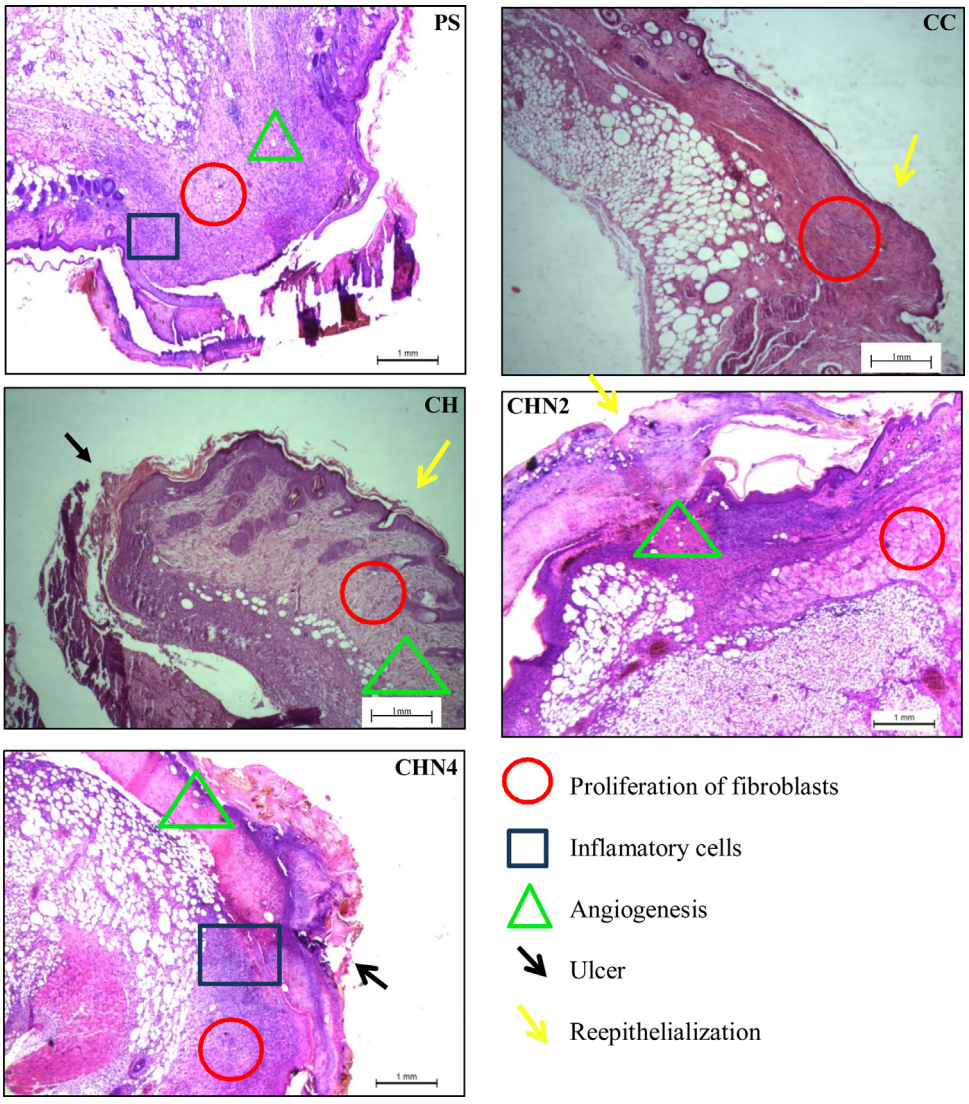

Fig. 5. Histological evaluation of cutaneous wound in mice on the 7th day of treatment with Physiological saline (PS), Collagenase *Clostridium histolyticum* (CC), Chistosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2), Chitosan Hydrogel with 4% Nerolidol (CHN4).

3.3. Wounds healing tests

3.3.1. Macroscopic analysis of wounds

CH is an active material for wounds healing and helps in the complex process of re-epithelialization. Some studies show that this material accelerates healing by stimulating the intense proliferation of fibroblasts and angiogenesis, besides preventing the development of infections (Du et al., 2012).

From biological and economic perspectives of this material, a deep evaluation of its real action on the healing of skin wounds was necessary. Therefore, the healing process of wounds treated with physiological saline (PS), Collagenase *Clostridium histolyticum* (CC), Chitosan Hydrogel (CH), Chitosan Hydrogel combined with 2 and 4% of Nerolidol (CHN2 and CHN4) was macroscopically observed. This made it possible to attest to the feasibility of using these materials in the wounds healing process (Fig. 4).

Fig. 4. Considering the macroscopic analysis of the cutaneous lesions in mice treated with physiological saline (negative control), Collagenase *Clostridium histolyticum* (CC) (positive control), CH and CH combined with NRL, was possible to verify the evolution of tissue injury repair, with the presence of bleeding in the groups PS, PC and CH, which is considered as detrimental to healing process since could compromise cell proliferation (Lucena et al., 2006).

On the 14th day, except for the groups treated with PS and CHN4, all the wounds were totally closed. In the first week of treatment the presence of exudate was observed specially in the PS, CH and CC groups. In the same period crust formation in all groups was verified. The crust protects the wound and contributes to cell proliferation (Silva et al., 2013). After 21 days, all groups showed the fully closed wound, indicating complete healing.

The diameter of the wound was measured using an analogical pachymeter and showed a difference among the groups on the 7th and 14th day of treatment. On the 1st day all mice showed a wound with 0.6 cm diameter and in the end of the experiment all animals presented a totally healed wound. Fig. 4 shows the pictures of the evolution of the wound in the mice during the treatment and additionally a bar graph promotes better visualization of the lesion diameter for all groups.

On the 7th day the group treated with CHN2 presented wounds with a smaller size as the wounds of the other groups, due to the intense proliferation of fibroblasts and collagen reorganization, which was confirmed by histological results. On the 14th day the groups treated with PS and CHN4 still presented wounds with an

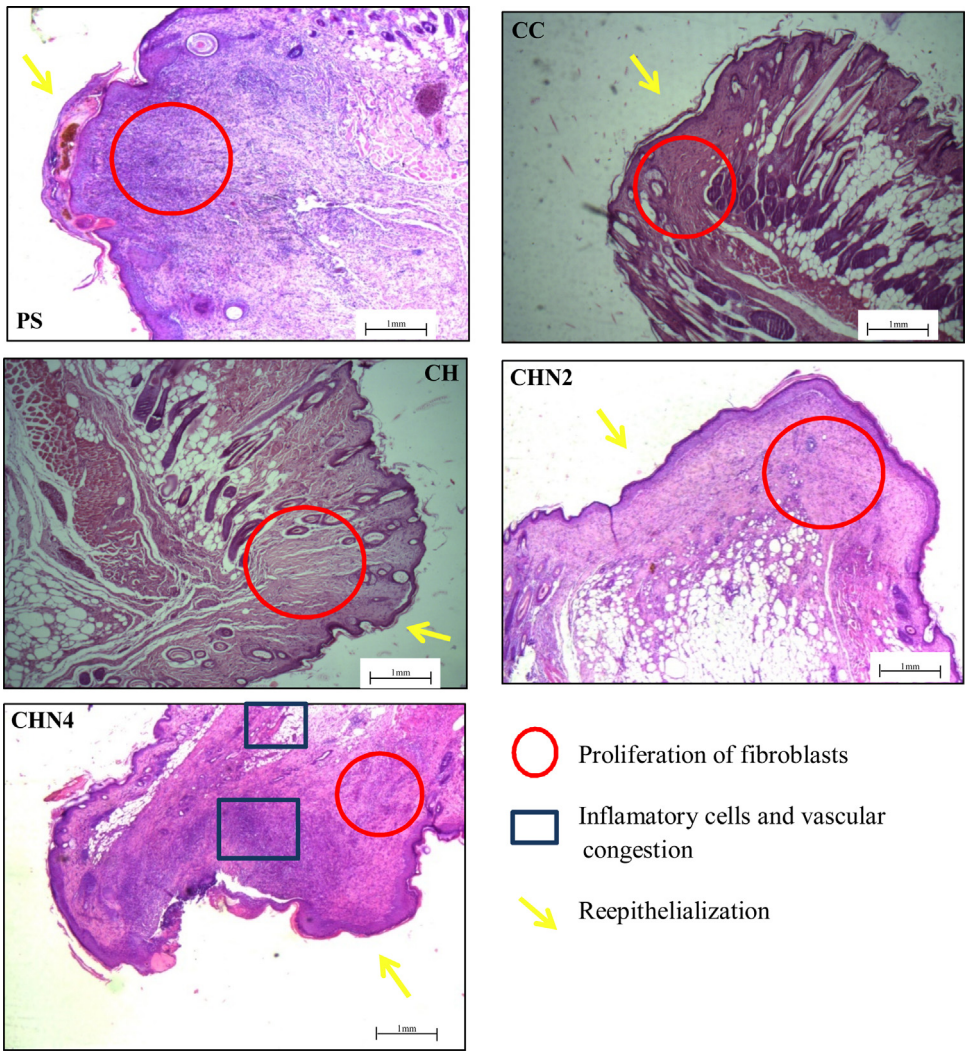

Fig. 6. Histological evaluation of cutaneous wound in mice on the 14th day of treatment with Physiological saline (PS), Collagenase *Clostridium histolyticum* (CC), Chistosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2), Chitosan Hydrogel with 4% Nerolidol (CHN4).

approximate size of 0.1 cm, which can be explained by the presence of moderate inflammatory foci.

Macroscopic analysis revealed that the CHN2 presented a good healing action. Besides this, the wound was smaller than in the group treated with commercial ointment (CC). Thus, the results showed superior properties of the CHN2 in comparison with the commercial standard. The chitosan hydrogel showed similar results to the ointment, and the groups treated with PS and CHN4 had a slower wound healing compared to the other groups, showing the presence of inflammatory cells, which confirm that there is a maximum concentration related with the specific biological activity (antimicrobial and healing properties) and a higher concentration of the substance can be unnecessary or even can cause harmful events.

3.3.2. Histological evaluation

Histopathological evaluation was performed on days 1, 7, 14 and 21 of the treatment. On the 1st day there were no structural changes observerd in the skin; the histological structure was comparable with normal skin (data not shown). There was the presence of epithelium with keratin, disordered connective tissue and dermal papillae. As required, these results reinforced that the animals didn't show contamination, injury or pre-existing infections in the wound area at the beginning of treatment.

Fig. 5 shows the results on the 7th day of treatment, and in all groups an intense fibroblast proliferation was observed.

Fig. 5. In the wounds treated with CH and CHN4 the presence of ulcer was observed. In the group treated with CH the presence of moderate to intense diffuse inflammatory focus was observed. Nevertheless, the presence of congestion or edema was not observed. The treatment with CHN4 provided wounds with intense inflammatory foci, presented diffusely throughout all ulcer extension. There was also the presence of edema and moderate congestion, and the discreet beginning of the re-epithelialization process was noted. The group treated with PS showed similar results as the group treated with CHN4, except for the presence of ulcer.

The groups treated with the PS, CH and CHN2 presented tissue re-epithelialization. This process was however less evident in the group treated with CH. The groups treated with CC and CHN2 demonstrated intense angiogenesis and also a proliferation of fibroblasts, with no edema, vascular congestion or even inflammatory focus, showing a better tissue reorganization.

On the 14th day, all groups showed intense angiogenesis. A slower healing was visualized in CHN4, with discreet edema, vascular congestion and with the presence of intense inflammatory focus with lymphocyte predominance. The re-epithelialization process was observed in all groups, being more discreet in CHN4 (Fig. 6).

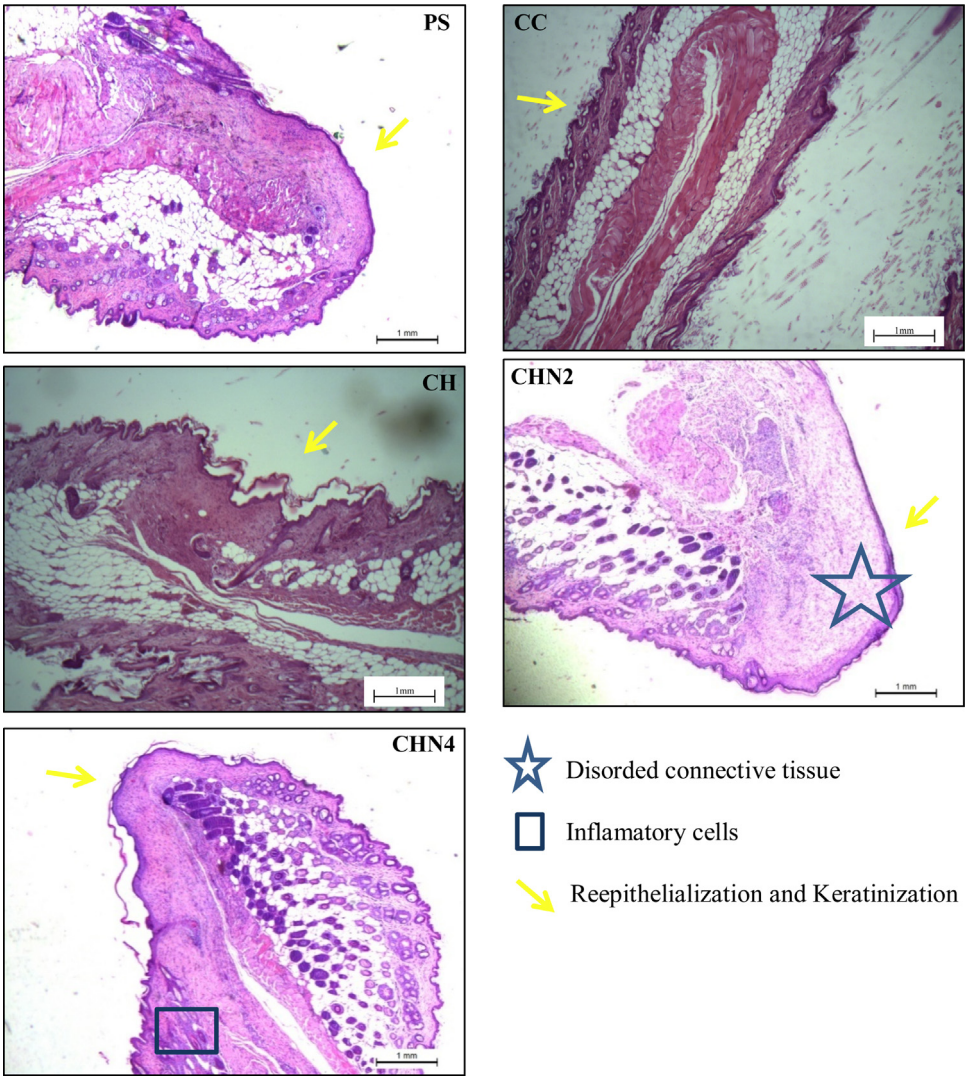

Fig. 7. Histological evaluation of cutaneous wound in mice on the 21st day of treatment with Physiological Saline (PS), Collagenase *Clostridium histolyticum* (CC), Chistosan Hydrogel (CH), Chitosan Hydrogel with 2% Nerolidol (CHN2), Chitosan Hydrogel with 4% Nerolidol (CHN4).

Fig. 6. In the groups treated with CH, CC and CHN2 the presence of edema and inflammatory focus were not detected, showing the reorganization of collagen with dense aspect without modifications. The group treated with CHN2 showed stratified squamous collagen, indicating once more the proper performance as healing agent.

In the group treated with PS, in addition to comments already mentioned, a slight congestion without new hair follicles was observed. Moreover, there was restructuring and reorganization of the epithelium and connective tissue.

The best results were obtained in the group treated with CHN2, with presence of re-epithelialization, better appearance of connective tissue, restructuring of connective matrix, intense angiogenesis and fibroblast proliferation. This formulation therefore proved to have great healing effects; even better than CC, which is considered a gold standard.

Fig. 7 shows the results obtained on the 21th day. The group treated with CHN4 did not present hair follicles or even dermal papillae. The beginning of reorganization of the dense disordered structure and intense angiogenesis in connective tissue was observed. Besides this, the complete re-epithelialization with presence of stratified squamous epithelium was observed. However, it was still verified the discreet presence of pontual inflammatory foci with the predominance of lymphocytes.

Fig. 7. The group treated with PS (negative control) showed similar results on the 14th day of treatment, although the group had a better organization of connective tissue, with the disordered dense structure becoming well defined. In the other groups inflammatory focus and edema was not observed. On the other hand, the presence of dermal papillae and re-epithelialization with keratinization of squamous epithelium was verified.

CHN2 showed better structuring of the connective tissue, with the formation of disordered dense connective tissue, and a greater number of dermal papillae compared to other groups. The healing process was complete for most groups, except for the CHN4.

The excellent properties of chitosan, including being hemostatic and a promoter of fibroblast proliferation, as well as showing good antibacterial activity, in stimulating the immune system and oxygen permeability and being non-toxicity, seem to have been potentiated/improved by nerolidol, demonstrating the synergic effect of these compounds (Azad et al., 2004; Berger et al., 2004; Jayakumar et al., 2011; Jin et al., 2007; Minami et al., 1999; Park et al., 2009).

4. Conclusion

The preparation of pure chitosan hydrogel or hydrogel combined with 2 or 4% of nerolidol is simple and fast, which makes this material commercially attractive. The presence of nerolidol in the hydrogel matrix was confirmed by characterization techniques. In the TG and DSC curves there were variations in the degradation profile, and the main absorption bands of these materials were observed in the infrared spectra. These changes firmly reasserted the combination of these materials. Through antimicrobial tests in vitro, it was proven that CH combined with nerolidol possesses greater inhibitory effect than chitosan or nerolidol alone. It also has an excellent action against *Staphylococcus aureus*. CHN2 proved to have a better healing effect compared to the gold standard (collagenase *Clostridium histolyticum*). Therefore, it is feasible to use CHN2 as healing agent, since it is simple and has a low cost, which makes this formulation economically attractive for biomedical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.07.037.

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