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Gelatin-based membrane containing usnic acid-loaded liposome improves dermal burn healing in a porcine model

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ABSTRACT

There are a range of products available which claim to accelerate the healing of burns; these include topical agents, interactive dressings and biomembranes. The aim of this study was to assess the effect of a gelatin-based membrane containing usnic acid/liposomes on the healing of burns in comparison to silver sulfadiazine ointment and duoDerme® dressing, as well as examining its quantification by high performance liquid chromatography. The quantification of the usnic acid/liposomes was examined using high performance liquid chromatography (HPLC) by performing separate in vitro studies of the efficiency of the biomembranes in terms of encapsulation, drug release and transdermal absorption. Then, second-degree 5 cm^2 burn wounds were created on the dorsum of nine male pigs, assigned into three groups ($n = 3$): SDZ – animals treated with silver sulfadiazine ointment; GDU – animals treated with duoDerme®; UAL – animals treated with a gelatin-based membrane containing usnic acid/liposomes. These groups were treated for 8, 18 and 30 days. In the average rate of contraction, there was no difference among the groups ($p > 0.05$). The results of the quantification showed that biomembranes containing usnic acid/liposomes were controlled released systems capable of transdermal absorption by skin layers. A macroscopic assay did not observe any clinical signs of secondary infections. Microscopy after 8 days showed hydropic degeneration of the epithelium, with intense neutrophilic infiltration in all three groups. At 18 days, although epidermal neo-formation was only partial in all three groups, it was most incipient in the SDZ group. Granulation tissue was more exuberant and cellularized in the UAL and GDU groups. At 30 days, observed restricted granulation tissue in the region below the epithelium in the GDU and UAL groups was observed. In the analysis of collagen though picrosirius, the UAL group showed greater collagen density. Therefore, the UAL group displayed development and maturation of granulation tissue and scar repair that was comparable to that produced by duoDerme®, and better than that produced by treatment with sulfadiazine silver ointment In addition, the UAL group showed increased collagen deposition compared to the other two groups.

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1. Introduction

Damaged tissue provides a potent culture medium for the growth of bacteria because of its temperature and moist, nutrient rich environment (Miyazaki et al., 2012; Muzzarelli, 2009). A factor of great importance for the successful treatment of lesions is their

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protection from the external environment with occlusive dressings. Collagen membranes have a great advantage in this area, especially in the treatment of burns, as their characteristic resorbability means no second surgical intervention is required for their removal, avoiding postoperative complications and patient discomfort (Boateng et al., 2008; Neel et al., 2012). However, collagen membranes, which have been in use since the late 19th century, can have poor mechanical strength, shape stability and low elasticity. Gelatin-based membranes, which have been employed in recent years, are a potentially useful biomaterial without some of the problems of collagen and are especially effective in the promotion and acceleration of granulation and epithelialization (Albuquerque-Júnior et al., 2009; Dantas et al., 2011) and also allow gradual drug release within target tissues (Dias et al., 2011). Gelatin membranes containing compounds that promote wound healing can be produced. A promising compound is usnic acid, one of the best studied of the various lichen compounds (Ahmadjian, 1993). It comes from the secondary metabolism of lichens (an association between fungi and algae) and has proven healing (Nunes et al., 2011), antimicrobial (Segatore et al., 2012) and antibiotic (Honda et al., 2010) effects. However, exploiting these therapeutic qualities is difficult because the metabolic lichens have unfavorable physicochemical characteristics, such as low water solubility. One way to overcome this is to use liposome-loaded usnic acid (UALs) combined with the gelatin membrane. Liposomes are artificially prepared membranous vesicles composed of natural phospholipids and cholesterol. Their structure is similar to the cell membrane in terms of hydrophilicity and lipophilicity, and are suitable for use as the carrier of drugs insoluble in water, such as usnic acid. It can be used to target the slow release of the drug, thereby reducing toxicity, and improving bioavailability. Liposomes are biodegradable, biocompatible, non-immunogenic and are therefore attracting increasing attention in medicine (Fan et al., 2013; Huanga et al., 2014). Previously, our group, Nunes et al. (2011), published a study of the effect of a collagen-based membrane containing usnic acid/liposome on the treatment of burns in rodents, and now, in this study, aim to evaluate the effect of a gelatin-based membrane containing usnic acid-loaded liposome in pigs, comparing two reference products used in burn treatment, silver sulfadiazine and DuoDerme®.

2. Materials and methods

2.1. Membrane preparation

The gelatin solution containing the liposomes/usnic acid was prepared using the "casting" method, in two steps. First a powdered gelatin solution containing acetic acid and a plasticizer (propylene glycol) was created and mechanically stirred for 24 h. At the same time, a lipid solution (phosphatidylcholine – Lipoid GMBH 75%) containing UA and an organic solvent (chloroform) was prepared and, through the solvent evaporation method using a rotary evaporator (24 h), a dry film was formed on the evaporator balloon. This was then resuspended in distilled water and the film detached from the balloon using mechanical agitation. Ultra sound is then used to make this into a solution. The two solutions were then mixed for 24 h using magnetic agitation, and finally for 30 min using ultra sound. This mixture was poured into glass plates and after evaporation of water a dry membrane was formed on the plate, which was cut into squares (5/5 cm) for use with the bioassay animals. Nunes et al. (2010) described the characterization of membranes.

2.2. HPLC analysis and method validation

The usnic acid was determined using an HPLC system that consisted of a degasser DGU-20A3, two LC-20AD pumps, a SIL-20A HT auto injector, a CTO-20A column oven, an SPDM20Avp photodiode array detector (DAD) and a CBM-20A system controller (Shimadzu Co., Kyoto, Japan). The analysis was performed using a Phenomenex Luna C18 analytical column of 150 × 4.6 mm (5 μm particle size). The mobile phase used was methanol–water: acetic acid 1% (90–10) with a flow rate of 1.0 mL min^{-1} and the injection volume was 20 μL. The detector was set at 280 nm for acquiring the chromatogram. The data were obtained using Shimadzu LC Solution software. The HPLC method was validated in respect of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness through procedures established by the International Conference on Harmonization guidelines (ICH, 2005). The linearity was obtained using a calibration curve at five different concentrations: 5, 10, 25, 50 and 100 μg mL^{-1}. The LOD and LOQ were calculated based on the standard deviation of the y intercept (σ) and the slope of the standard calibration curve (S). LOD was calculated by the equation 3.3 σ/S and the LOQ also examined by the equation 10 σ/S. The precision of the method was investigated in relation to repeatability (intraday precision) and intermediate precision (interday precision). Six samples of usnic acid (100 μg mL^{-1}) were injected on the same day to evaluate repeatability. The intermediate precision was evaluated by analyzing usnic acid samples in the same concentration on different days and by a different analyst. The precision was expressed as relative standard deviation (RSD%). Accuracy was evaluated by using solutions containing three known concentrations (5, 50 and 100 μg mL^{-1}) of usnic acid analyzed in triplicate. Accuracy was calculated as percent recoveries of response factor (area/concentration). The ruggedness of the method was evaluated by changing the mobile phase flow rate and using different analytical columns.

2.3. Encapsulation efficiency of membranes (EE%)

The EE% was determined by dissolving a membrane segment with an area of 7 cm^2 in 10 mL of methanol under constant stirring (250 rpm) for 12 h to allow all entrapped usnic acid to be in solution. After this procedure, the solution was filtered through a 0.22 μm membrane filter and analyzed by HPLC. The samples were prepared through three different membranes and analyzed in triplicate. The EE% was calculated according to the equation:

$$EE\% = \frac{C1}{C2} \times 100 \quad (1)$$

where, C1 is the content of usnic acid present in the membrane and C2 is the usnic acid amount initially used to prepare the membrane.

2.4. *In vitro* membrane release

The usnic acid membrane release was performed with a vertical automated Franz diffusion cell (MicroettePlus Multi-Group®, Hanson Research Corporation, Chatsworth, CA, USA) operating at 32 ± 0.5 °C. The diffusion area was 1.76 cm^2 and the receptor chamber volume was 7.0 mL. A dialysis membrane (MWCO = 12 kDa, Sigma-Aldrich), pre-hydrated for 8 h was fixed between the donor and the receptor compartments. Sink conditions were maintained using a receptor medium composed of 2% (v/v) DMSO. Sample aliquots (2 mL) were collected each 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 18 and 24 h and analyzed by HPLC.

2.5. Transdermal absorption of usnic acid from the membranes

The transdermal absorption studies were conducted using porcine ear skin as the membrane. The membrane samples were donated from a regional slaughterhouse (Bento Gonçalves, Brazil).

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