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Short communication

Caffeic acid skin absorption: Delivery of microparticles to hair follicles

Lia Carolina Oliveira dos Santos [a], Caroline Magnani Spagnol [a], Antonio José Guillot [b], Ana Melero [b,*], Marcos Antonio Corrêa [a]

[a] Department of Drugs and Medicines, São Paulo State University (UNESP), School of Pharmaceutical Sciences, Rod. Araraquara Jau Km 1- Campus Ville, CEP:14800-903 Araraquara, SP, Brazil
[b] Department of Pharmacy and Pharmaceutical Technology, Universitat de València, Valencia, Spain

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ABSTRACT

Caffeic acid (CA) is a polyphenol that can be found in a wide range of vegetal dietary sources. It presents a remarkable antioxidant potential, but what is more interesting from the therapeutic point of view is, that it has demonstrated *in vitro* antimicrobial properties. Folliculitis is a common skin condition, usually caused by a bacterial or fungal infection, in which hair follicles become inflamed. A typical challenge in dermal application when the actives diffuse passively through the skin in a quick manner, as it is the case of CA, is to provide the effective concentration of the compound at the target site for the sufficient time to finalize the treatment adequately and reduce the possibility to trigger systemic side effects. To achieve this goal, it is necessary to appropriately design the drug delivery system. In this case, we leverage the ability of microparticles to accumulate into the hair follicles to design O/W-emulsions containing CA-loaded controlled-release microparticles. Two different emulsion types containing CA were prepared, one containing free CA and the other containing microencapsulated CA. Traditional and differential tape stripping techniques were performed to investigate drug distribution within the different skin layers and into the hair follicles. The Tape stripping results demonstrated that the tapes S3-S5 and S6-S10 presented a higher total amount of CA. The strips are collected and extracted in groups to assure the extraction of quantifiable amounts of drug. Samples S11-15 and S16-20 show a decrease in the amount of quantified CA, as it was expected. Thus, it can be seen that the amount of active decreases while the stratum corneum depth increases. The retention studies demonstrated that, the microparticles tend to produce a more homogeneous distribution of CA, within the stratum corneum and a higher retention into the hair follicle, which can be attributed to their size and uniformity. Besides, MPs present an additional advantage because they guarantee a continuous release of CA in the target for a prolonged period, allowing the treatment of folliculitis with a single dose until the MPs are removed from the hair follicle by its natural regeneration process or particle depletion of CA.

1. Introduction

Caffeic acid (CA) (3,4-dihydroxycinnamic acid) is one of the most widely distributed hydroxycinnamate and phenylpropanoid metabolites, being thus a polyphenol that can be found in many dietary sources of plant origin, including coffee drinks, blueberries, apples, and cider (Clifford, 2000). In addition to the notorious antioxidant potency of CA, *in vitro* studies demonstrated the antimicrobial action of propolis against different pathogenic microorganisms of the mouth (Huang and Ferraro, 1992; Marcucci, 1996; Menezes, 2005). It is also known as a carcinogenic inhibitor (Greenwald, 2004, Sanchez-Moreno et al., 2000) and can contribute to the prevention of atherosclerosis and other cardiovascular diseases (Vinson et al., 2001; Zucheto et al., 2011; Magnani et al., 2014).

Acne is the most common among skin diseases and the following etiopathogenic factors are prominent: sebum production by the sebaceous glands, follicular hyperkeratinization, release of inflammatory mediators in the follicle and adjacent dermis and bacterial colonization of the follicle (Barbosa et al., 2014).

* Corresponding author at: Department of Pharmacy and Pharmaceutical Technology, Universitat de València, Av. Vicent Andrés Estellés, 46100 Burjassot, Valencia, Spain.
E-mail address: ana.melero@uv.es (A. Melero).
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The three major microorganisms isolated from the skin surface and sebaceous gland ducts of individuals with acne are *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Malassezia furfur*, the first being certainly the most importante (Barbosa et al., 2014). *P. acnes* is a gram-positive, anaerobic bacillus that is involved in the inflammatory response of the pathogenesis of acne (Matsuchita and Matsuchita, 2015). It is predominant in the sebaceous region of the skin, located in the hair follicle, being transported to the surface of the skin by the sebaceous flow. It metabolizes fractions of sebaceous triglycerides and occurs more in adolescence, being rare in childhood. It is not an infectious bacterium, but it is one of the main factors in the development of acne (Manca et al., 2014).

For many years, antibiotic therapy has been a form of treatment, however, the adverse reactions caused by the medications end up making the treatment unpleasant, in addition to reported cases of decreased sensitivity to antibiotics (Matsuchita and Matsuchita, 2015). The topical drugs class include retinoids (Rancan and Vogt, 2014), benzoyl peroxide, antibiotics, alpha-hydroxyacids, azelaic and salicylic acids and corticosteroids, and systemic treatment consists of oral antibiotics (Barbosa et al., 2014).

The use of natural products in dermatology is becoming increasingly common due to the increased resistance of bacteria to synthetic antibiotics and the active principles of medicinal plants become a new option as antiseptics and antimicrobials (Matsuchita and Matsuchita, 2015). Several propolis components were analyzed in different countries, with CA, phenolic esters of CA and flavonoids responsible for the antibiotic power of this resin. The antimicrobial activity of some active substances is widely explored in the cosmetic segment in the form of products with various purposes (Barbosa et al., 2014). It is believed that compounds such as flavonoids, CA, benzoic acid, cinnamic acid, appear to act on the membrane or cell wall of the microorganism, causing functional and structural damage. From these data, it is suggested to formulate less aggressive cosmetic formulations, but to provide the efficacy of drugs frequently used for acne (Matsuchita and Matsuchita, 2015).

Acne treatment is primarily aimed at minimizing the physical and aesthetic discomforts of acne inflammations, preventing and reducing marks and scars which can lead, depending on the intensity, psychological, emotional and social damages (Manca et al., 2014).

In the past, the trans-follicular route was only a secondary route of transdermal penetration (Illel et al., 1991). However, in the decade of the 90′s, it was demonstrated that percutaneous penetration of several drugs was greater in hairy mice compared to hairless mice (Spagnol et al., 2017).

Advances in nanotechnology have opened new opportunities for the delivery of molecules to the hair follicle (HF) as they can accumulate into these structures releasing drugs for an extended period, since both hydrophilic and lipophilic compounds can be incorporated in these delivery systems (Gilliam et al., 1998). As HF represents an invagination, or extension, of the epidermis, it offers an increased available surface area, much greater than initially assumed. In addition, this epidermis is much thinner and the region is surrounded by numerous blood capillaries and immunocompetent cells, which contributes to drug absorption and / or the production of an immune response (Lademann et al., 2009; Mathes et al., 2016). In 2006, Lademann et al. (2009) also found that HF act as a long-term reservoir of these particles for up to 10 days. This finding means that, after accumulation on the HF the drugs can be released over time and continuously diffused into the surrounding follicular and perifollicular cells or through the capillary walls to reach the bloodstream. Targeted delivery of medications through HF can become very advantageous as it covers a wide range of different applications ranging from cosmetic products for acne treatment to non-invasive transcutaneous vaccination, including treating cutaneous disorders such as atopic dermatitis, folliculitis and even psoriasis (Morais, 2006; Lademann et al., 2009). For this work, microparticles (MPs) containing CA have been designed for the treatment of folliculitis and incorporated into a semisolid formulation to facilitate its topical application. In this case, oil/water (o/w)-emulsions were selected to formulate both, the MPs and free CA as a control. Emulsions present several advantages over other semisolid formulations, such as an easy scattering, possible penetration enhancement, protection against unstable compounds, tunable release of drugs and absorption, with the consequently modulation in the activity of the incorporated drugs (Shah et al., 1998).

Taking into account the characteristics of the skin and the demonstrated antimicrobial efficacy of CA, drug distribution studies within the different skin layers and structures of this drug are of great interest. In the present work, we perform these studies through the techniques of tape stripping and differential stripping of CA incorporated in MPs.

The tape stripping technique is widely used and accepted to study the kinetics and penetration of active substances in the skin, allowing to determine the location and distribution of substances in the stratum corneum (SC). Especially in the last decades, it has become one of the main investigative techniques used in studies of topical bioavailability and bioequivalence, being proposed by the FDA in 1998 (Pinkus, 1951; Caron et al., 1990; Benfeldt et al., 1999; Escobar-Chavez et al., 2008; Bettoni, 2009). The method of evaluation of cutaneous penetration can be performed in two ways, *in vitro* or *in vivo*. The *in vitro* method has a number of advantages over *in vivo*, since they can be used on human skin or on other species skins, many replicates can be made, there is no use of live animals, thus avoiding ethical and still several pharmaceutical forms can be studied. The limitation attached to the *in vitro* approach is that the sink conditions generated by the blood flow in the dermis are not fully reproduced (Spagnol et al., 2018).

The Differential Stripping method is the most straightforward technique to determine follicular uptake quantitatively. For this study, pig ear skin is used because it has been evaluated as a valuable *in vitro* model, being known as the “gold standard” since it is considered superior to human skin because HFs in the human skin contract immediately after excision, whereas the cartilage in the porcine skin prevents the contraction of the traction fibers and, therefore, the subsequent closure of the HFs. Anatomically, the HFs of human and porcine are similar in several aspects, such as SC density and follicular diameter. Thus, the pig ear is attributed to this type of study and only ears with immaculate skin surface (no abrasions, inflammation, etc.) should be used (Lademann et al., 2009). The term ‘Differential Stripping’ was first introduced in 2005 by Teichmann et al., and described the combination of the tape stripping technique with cyanoacrylate skin surface biopsies (removing the follicular cast). However, the method was rather a qualitative analysis than a quantitative way of determining follicular penetration. In 2014, Raber et al. modified and optimized this method, and ultimately obtained a protocol which made it feasible to fully quantify follicular uptake of particles (Lademann et al., 2009).

In this study, we develop two emulsion types, one containing free CA dispersed in the oil phase of the emulsion and incorporated in MPs, as a sustained release delivery system to target the hair follicles. Drug distribution studies were performed by the two avobe mentioned techniques in human and porcine skin respectively to demonstrate that the formulated particles do improve the drug accumulation into the target tissue, which is the hair follicle.

2. Material and methods

2.1. Materials

Skin samples: abdominal human skin samples obtained from surgical corrections after previously signed informed consent by the patients at the Wuiron Clinic, Valencia. Porcine skin was obtained from the Scientific Support Center for Research of the University of Valencia. The use of animal and human tissue was approved by the Research Ethics Committee of the Universitat de Valencia, Spain under the Protocol number: H1462978691586 and the sample obtention and further treatment followed the current European Law; Chemicals: Tesafilm® kristall-klar (Tesa, 33 m × 19 mm, cut to 30 × 19 mm sections) was obtained from Tesa AG, Hamburg, Germany; UHU superglue (UHU, blitzschnell Pipette) was kindly provided by UHU GmbH & Co, KG, Bühl/Baden, Germany; Parafilm Sealing Film – 50 mm (2″) Film Width × Roll Length 75 m (Madrid/Spain); Glass Beads (MERCK® - Madrid/Spain); Composition of the mobile phase: distilled water, methanol grade CLAE (MERCK® - Madrid/Spain), Acetic acid (MERCK® - Madrid/Spain); Composition of the emulsion: Cetearyl Alcohol and Sodium Cetearyl Sulfate (Mapric® - Brasil), Glyceryl Stearate (CUTINA® GMS V - BASF® - Brasil), Ethylhexyl Stearate (BASF® - Brasil), Glycerin (Mapric®), EDTA Na_2 (Brasquim®), Cafeic acid (Nanjing Zelang Medical Technology); Microparticles composition: Cafeic acid (Nanjing Zelang Medical Technology), Chitosan medium molecular weight (Sigma Aldrich®- Brasil); Acetic acid (Synth - Brasil); polysorbate 80 (Synth – Brasil).

2.2. Methods

2.2.1. Preparation of formulations containing CA

Emulsions containing CA were prepared at laboratory scale, one of which contain free CA and the other microencapsulated CA. Their composition is described in Table 1. The MPs are composed of 10% CA and were obtained by spray drying technique as described by Spagnol and coworkers (2018). A dispersion of chitosan with medium molecular weight (Sigma- Aldrich!) was prepared at 0.5% (w/v) in 1% (v/v) acetic acid. To this dispersion, a mass of CA equivalent to 30% of the chitosan dry weight and 1% of polysorbate 80 was added for CA solubilization in the dispersion. The dispersion of chitosan [0.5% (w/v)] with CA was atomized in BUCHI-290 Mini Spray Dryer. The following parameters were programmed into the equipment: air inlet temperature, 180 °C; pump flow, 10%; atomizing air flow, 450 NL/h; aspirator efficiency, 90%; and air outlet temperature, 80 °C. (Spagnol et al., 2018).

2.2.2. Tape stripping

The assay was performed using abdominal human skin samples obtained from surgical corrections by approval of the Research Ethics Committee of the Universitat de Valencia, Spain under the Protocol number: H1462978691586. Excess fat and connective tissues were removed, and the samples stored in a freezer at −26 °C for less than three months. The skin was placed on a glass blade with the outside facing upwards and an aluminum mold was placed over it, leaving the application area uncovered. Two tests were performed. In the first one, the formulation containing free CA was applied to delimited area of skin and in the second, the formulation containing the CA encapsulated in the MP, both in the concentration of 0.5% CA. Thereafter, the system was incubated at 32 °C for two hours. Each test was performed in triplicate. (see Fig. 1).

Table 1
Composition of the emulsion.

Emulsion with free CA		Emulsion with microparticles of CA	
Inci Name	%	Inci Name	%
Cetearyl Alcohol	8.0	Cetearyl Alcohol	8.0
Sodium Cetearyl Sulfate	1.5	Sodium Cetearyl Sulfate	1.5
Glyceryl Stearate	1.0	Glyceryl Stearate	1.0
Ethylhexyl Stearate	3.0	Ethylhexyl Stearate	3.0
Glycerin	3.0	Glycerin	3.0
EDTA Na_2	0.1	EDTA Na_2	0.1
Water	q.s.p. 100	Water	q.s.p. 100
Free CA	0.5	Microparticles with CA	5.0

The technique is characterized by being minimally invasive in which the SC is removed after sequential applications of adhesive tapes, allowing the quantification of active present in the first layer of the skin (Pinkus, 1951; Caron et al., 1990). The removed tape strips contain not only information about the amount of corneocytes and the penetrated substances but also about the distribution of the substances in the different depths of the SC (Baleeiro et al., 2013).

Tape stripping experiments are relatively easy and simple to perform; however intrinsic or extrinsic parameters may influence the amount of active and SC removed by the tape. It is generally assumed that 10 to 20 adhesive tapes are required for complete removal of the SC and that the first 10 tapes may exhibit 90% of the concentration of the active present in that layer, so that the subsequent tapes contribute in less of 5% (Pinkus, 1951; Benfeldt et al., 1999; Bettoni, 2009).

Variability is avoided by performing replicates using skins from the same donor. The CA present in each sample is extracted from the strips by the use of an acidified ethanol: water (40: 60, v/v) extractive solution under stirring for a period of 24 h and subsequently quantified by HPLC through the method validated by Spagnol and coworkers (2015). A mass balance study was previously done extracting all samples, the rest of the skin and all materials in touch with the formulation, to assure the complete extraction of CA by this method. A recovery of 93.7 ± 2.34 was obtained. The tape-stripping data represent the drug distribution within the SC and gives an insight into the concentration gradient of CA through this layer, which is the limiting barrier for drug diffusion. The experiments were performed in triplicate.

2.2.3. Differential stripping

In order to analyze the extent of active penetration into the hair follicle, the differential tape stripping technique is used in the external auricle of porcine ear skin. The first step of this protocol entails applying the formulation onto a predetermined area on the outer side of auricle pig ear. After a predetermined incubation time under constant temperature conditions, subsequent tape strips are taken in order to clean the skin surface and remove the SC layer by layer like described in Section 2.2.2. (see Fig. 1).

To analyze the extent of follicular drug penetration, cyanoacrylate skin surface stripping is performed. This step implies the application of superglue to the pretreated skin area covered by a tape strip. Upon polymerization of the glue, the tape strip is quickly peeled off, removing the entire follicular cast.

For quantification and mass-balance purposes the substance of interest is extracted from the tape strips, cyanoacrylate biopsies, as well as all application devices, and skin rest are analyzed for drug content via an analytical method of choice (Lademann et al., 2009).. The experiments were performed in triplicate.

3. Results and discussion

3.1. Tape stripping

The first strip is usually discarded, as n the surface of the skin there is an excess of formulation that is difficult to be completely

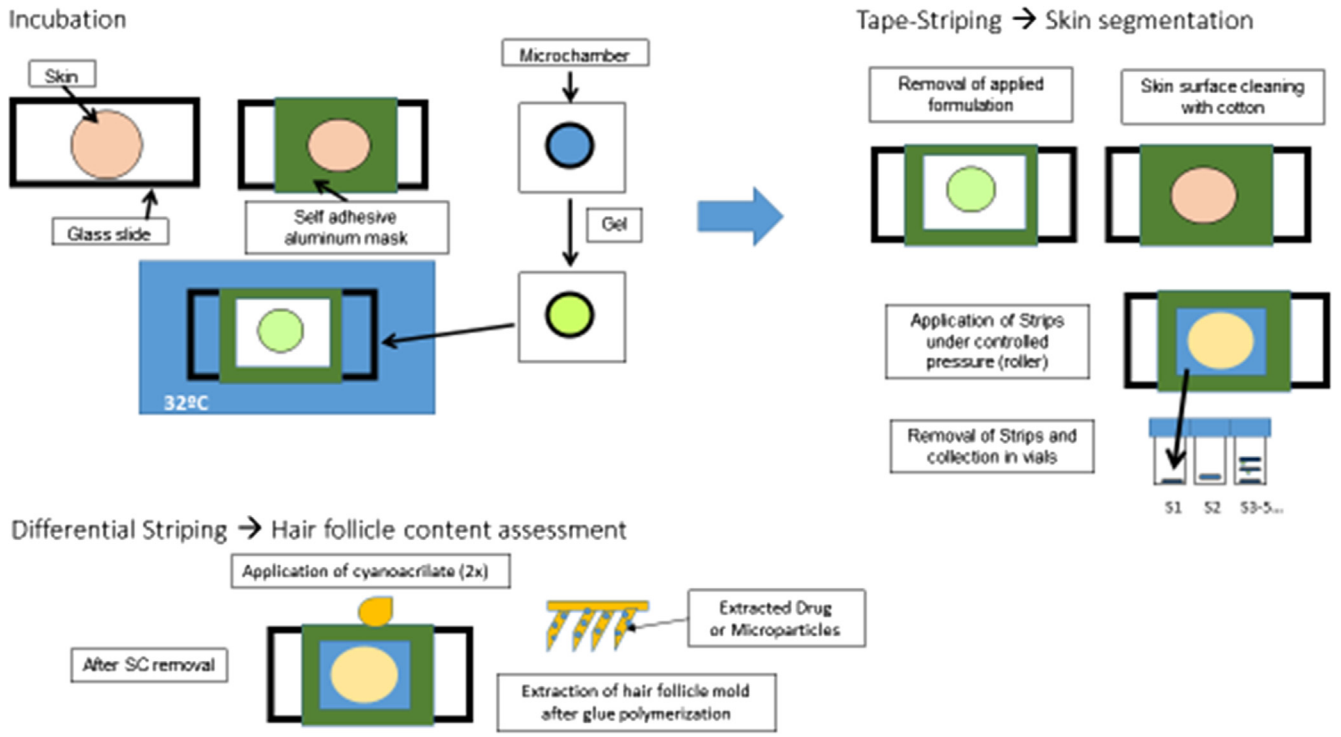

Fig. 1. Schematic Incubation, tape-stripping technique and differential-stripping technique.

clean without damaging the surface. This is the case in the present work and because of that (S1) was discarded.

Tape S2 shows a decrease in the total amount of CA extracted from the sample. Samples S3-S5 and S6-S10 show a higher total amount of sample, but it should be taken into account that this samples are the summatory of three strips. The strips had to be collected and extracted in groups because the amount of drug present in each strip is low and after dilution in the extraction medium, the concentration is too close to the detection limit to assure a reliable quantification.

Samples S11-15 and S16-20 show a decrease in the amount of quantified CA, as it was expected. Thus, it can be seen that the amount of active decreases while the SC depth increases.

The average amount of CA found in each tape when the tape stripping was performed with the emulsion containing free CA was: tape 1 = 3.877 μg; tape 2 = 1.79 μg; tapes 3–5 = 3.13 μg; tapes 6–10 = 5.04 μg; tapes 11–15 = 1.78 μg; tapes 16–20 = 1.03 μg. The amount of CA in the dermis and epidermis was also founded: 47.60 μg. The average amount of CA found in each tape when the tape stripping was performed with the emulsion containing CA-MPs was: tape 1 = 2.56 μg; tape 2 = 1.76 μg; tapes 3–5 = 2.39 μg; tapes 6–10 = 2.01 μg; tapes 11–15 = 1.32 μg; tapes 16–20 = 1.13 μg. The amount of CA (μg) in the dermis and epidermis was also founded: 2.88 μg.

These results are presented in Fig. 2. A comparative analysis between the studies performed with free and microparticulate CA, shows that the amount of active when incorporated in the MP is lower. However, there is a smaller variation of values between the tapes, what indicates a more homogeneous distribution of CA within the SC. The amounts of CA (μg) present in the SC formulated as a free CA emulsion vary between 1.03 and 5.04 μg, what clearly show significant oscillations compared to CA formulated in the emulsion containing MP (1.13 to 2.56 μg) (ANOVA, p = 0.5). This fact shows a better control not only in drug release but also in drug distribution within the tissue.

Although a higher amount of CA in the epidermis dermis was recovered when CA was not encapsulated, what was expected because the free drug is more readily bioavailable. CA-MPs, are drug delivery systems, which can be more retained on the SC releasing the drug in a controlled and prolonged way the active in the deeper layers of the skin. Thus, the release of CA contained in the MP, under the same conditions as an emulsion with free CA, leaves the SC towards the epidermis dermis.

3.2. Differential stripping

The present study showed a tendency of greater retention of the MPs in the hair follicle when compared to the study done with the free CA, since the amount of CA found in the follicle in the study with MPs is larger (Fig. 3).

The total amount of CA found in follicles when the differential stripping was performed, in triplicate. The values obtained from the emulsion containing free CA were: Follicle 1 = 0.84 μg; follicle 2 = 1.13 μg; follicle 3 = 1.15 μg. The amount of CA in the dermis and epidermis was: Epidermis and Dermis 1 = 9.57 μg; epidermis and dermis 2 = 15.55 μg; epidermis and dermis 3 = 9.20 μg.

The total amount of CA found in follicles when the differential stripping was performed, in triplicate, with the emulsion containing MP-CA was: Follicle 1 = 1.76 μg; follicle 2 = 1.72 μg; follicle 3 = 1.57 μg. The amount of CA in the dermis and epidermis was also founded: Epidermis and Dermis 1 = non-detectable amounts; epidermis and dermis 2 = 1.42 μg; epidermis and dermis 3 = 1.58 μg.

These results are presented in Fig. 3.

Follicular penetration is an important and promising pathway for dermatotherapy. Innovative cosmetic formulations are developed to optimize the penetration and storage of topically applied molecules. These approaches focus primarily on nano or micro technology. In order to observe the follicular penetration, it is interesting to carry out retention studies with formulations of emulsion type, since their higher viscosity allow the accumulation of is the MPs containing the drug inside the follicle. In the case of solutions, what are the most studied samples concerning follicular penetration, an easy entrance and exit of the follicle would be expected, rather than retention, as desired.

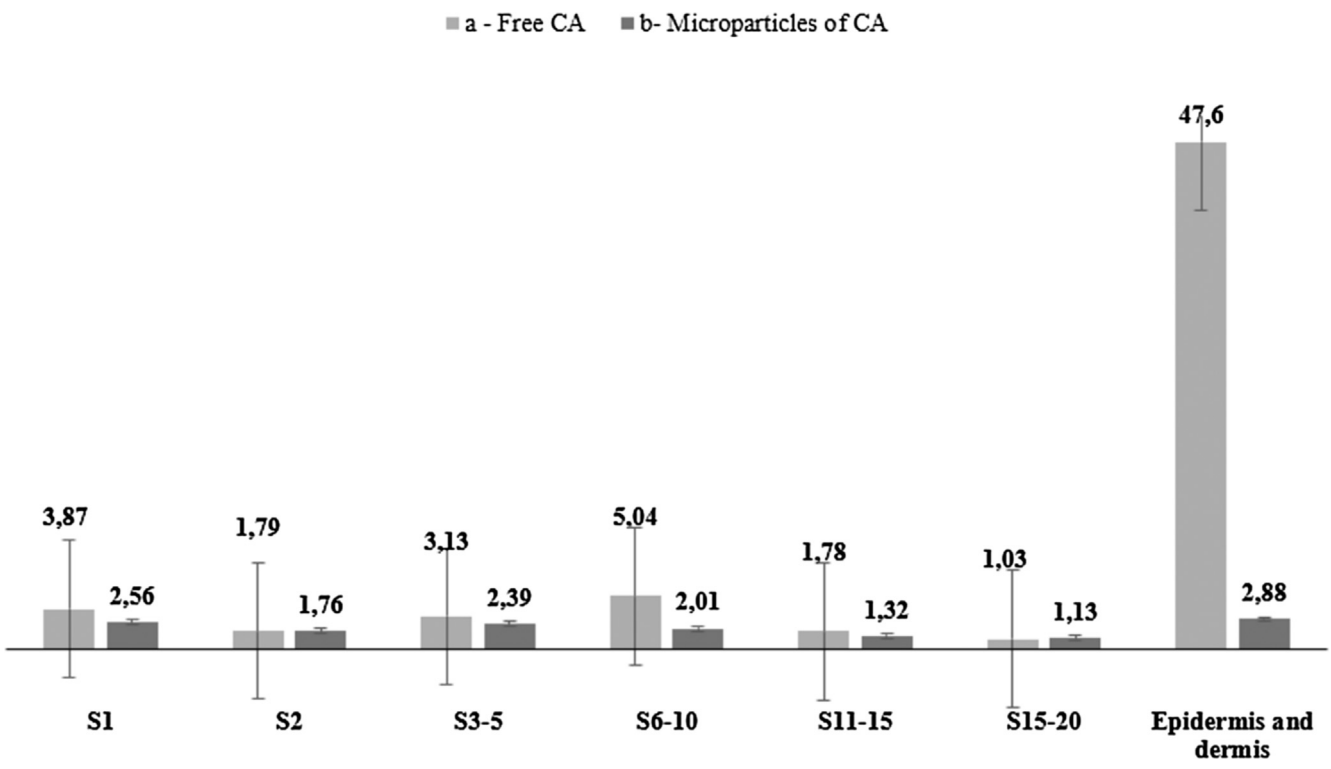

Fig. 2. Representative graph of the amounts of AC present in each layer of the stratum corneum and epidermis and dermis. a. Tape Stripping with emulsion with CA free; b. Tape Stripping with emulsion with microparticles of CA.

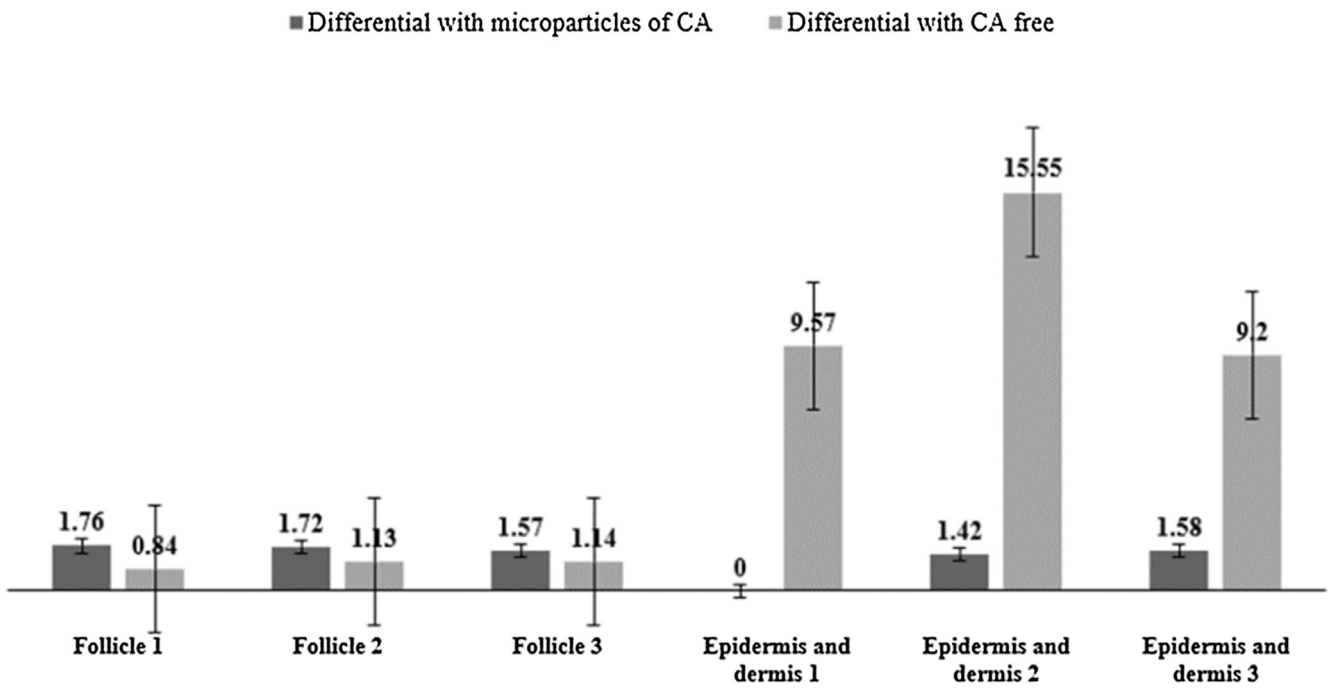

Fig. 3. Representative graph of the amounts of CA present in the follicle (Sum of the quantity of CA found in follicles in differential stripping) and epidermis and dermis of pig ear skin.

Considering the results obtained, the MP penetration efficacy is higher compared to the free CA formulation, as expected. About the greater tendency of retention in the hair follicle when the CA is incorporated into MP, it can be seen in Fig. 3 that the amounts of CA retained in the follicle from the MP emulsion is 1.68 ± 0.07 and from the emulsion containing free CA 1.04 ± 0.13. Through ANOVA analysis of variance a statistically significant difference between the values, considering the value of $p < 0.05$, was found.

According to previous works, the ability of micro and nanoparticles to accumulate into the hair follicles is mainly determined by their size (Ourique et al., 2011). That is why the particles of this work were already designed with the optimal size, according to

the morphology studies using photomicrographs obtained by scanning electron microscopy performed by Spagnol and coworkers (2018). In these studies, the samples were covered with a thin layer of gold photomicrographed at 10.000× magnification with an electron beam of 2.0 kV using the high-resolution scanning electron microscope S-4800 (Serial Number: HI-9269-0009) and it was found that the diameter of MP ranged from 1 to 5 μm and they are spherical and had a smooth surface, which is ideal for controlled release. Therefore, we demonstrate through this work that the designed MPs are suitable to target the hair follicle for the treatment of folliculitis. In addition, these structures offer the advantage over the free CA-emulsion, of guaranteeing a constant release of drug in these structures, allowing the reduction of applications, as it can be assumed that the drug will be released over the whole hair regeneration period, which is of about 15 days (Lademann et al., 2009). The previous permeation studies showed that the MP used have a flow of J = 0.92 ± 0.24 mg/cm^2/h and a lag time of T = 4.04 ± 0.28, which shows that, the emulsion containing MP, in addition to promoting a slower flow of AC, also took longer to begin to permeate. Therefore, as CA is retained in the MP formulation in a more efficient manner, thus, it can also be expected that the permeation profile is also more controlled (Spagnol et al., 2018). It was also demonstrated increased lag time and a reduction in the CA flux in the emulsion systems. This can be explained by the viscosity of the systems proving that viscosity is one of the factors that affect the flow of the permeant (Berrozpe et al., 2013). The drug in MP will therefore stay in contact with the skin, for a longer time, thus improving its antimicrobial directly on the target site, as desired when designing the formulation (Spagnol et al., 2018).

Previous drug release works demonstrated that the percentages of CA release after 30 min from an emulsion containing 0.5% active ingredient was high compared to the same formulation observed over 2 h but containing 1% encapsulated CA. Furthermore, the emulsion containing 0.5% CA showed only a small change after the 4 h evaluation, meaning that the dose was depleted (Gilliam et al., 1998). On the contrary, after the same time, the encapsulated CA-emulsion had still not reached an asimpthote.

Besides, the hair follicles present a thinner SC, thus facilitating the drug access to the infected area and to the epidermis beneath, offering the possibility to treat conditions affecting the area below the hair follicle as well.

4. Conclusions

From the present work it can be concluded that MPs deliver CA providing a more homogeneous distribution through the SC and are more retained in the hair follicle because of their size and uniformity, than free CA. Besides, MPs present an additional advantage because they guarantee a continuous release of CA in the target for a prolonged period, allowing the treatment of folliculitis with a single dose until the MPs are removed from the hair follicle by its natural regeneration process or particle depletion of CA.

Declaration of interest

The authors declare that they have no actual or potential conflicts of interest with this work.

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