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Research paper

Improved efficacy in the treatment of contact dermatitis in rats by a dermatological nanomedicine containing clobetasol propionate

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ABSTRACT

We developed a dermatological nanomedicine containing clobetasol propionate-loaded nanocapsules and evaluated its efficacy in a model of contact dermatitis after topical administration in rats. Hydrogels containing clobetasol propionate-loaded lipid-core nanocapsules or nanoemulsion (HG-CP-NC and HG-CP-NE, respectively) were prepared to evaluate the influence of the polymeric wall. They presented adequate pH values (5.50–6.50) and drug content (0.5 mg g^{-1}) and their rheograms exhibited a non-Newtonian pseudoplastic behavior. The best *in vitro* drug release control was obtained for HG-CP-NC (1.03 ± 0.11 μg cm^{-2} h) compared to the HG-CP-NE (1.65 ± 0.19 μg cm^{-2} h) and the hydrogels containing nonencapsulated drug (HG-CP) (2.79 ± 0.22 μg cm^{-2} h). A significant increase in NTPDase activity was observed in lymphocytes for the group treated with 0.05% HG-CP-NC every other day compared to the group treated with 0.05% HG-CP every day using the *in vivo* model of contact dermatitis. The nanoencapsulation of clobetasol in nanocapsules led to a better control of the drug release from the semisolid nanomedicine and provided better *in vivo* dermatological efficacy.

1. Introduction

Topical corticosteroids have been widely used to treat skin diseases. Their clinical effectiveness in the treatment of psoriasis and atopic dermatitis is related to their vasoconstrictive, anti-inflammatory, immunosuppressive, and antiproliferative effects [1]. However, the use of topical glucocorticoids, after systemic and topical administration, is limited due to their adverse effects, such as skin atrophy, steroid acne, hypopigmentation, and allergic contact dermatitis [2,3]. Currently, researches have been focusing mainly on the development of strategies to improve the benefit-risk ratio of glucocorticosteroids [4–6]. With a view to decreasing the adverse effects, nanotechnology collaborates through the reduced particle size of its nanosystems, improving the absorption and therapeutic concentration of the drug in the target tissue, allowing reproducible and long-term release of the drug at the target site, reducing the frequency of drug administration, and improving its pharmacokinetics [7]. Clobetasol propionate (CP) is a super high-potency dihalogenated corticosteroid used for the treatment of skin disorders such as atopic dermatitis and psoriasis [8,9].

Polymeric nanocapsules are vesicular nanocarrier systems in which an oily phase is confined in a cavity surrounded by a thin polymeric membrane [10,11]. The preparation of semisolid formulations containing polymeric nanostructured systems for cutaneous application has been studied recently, aiming to control the release of some active substances, to improve their photostability [6,12–16], and to promote the drug penetration in the stratum corneum [15]. Moreover, the small particle size of the nanocarriers ensures close contact with the stratum corneum [17].

Milão et al. [12] showed that hydrophilic gels (Carbomer 940®) containing diclofenac-loaded nanocapsules present non-Newtonian behavior and plastic properties. Intact nanostructures were observed in gels by freeze-fracture electron microscopy after 3 months of storage at room temperature. Jiménez et al. [13] demonstrated that the skin accumulation of octyl methoxycinnamate after the application of an oil-in-water emulsion containing its free form was significantly lower in comparison with the formulation containing octyl methoxycinnamate-loaded nanocapsules. The inclusion of octyl methoxycinnamate-loaded nanocapsules in sunscreen formulations decreases its accumulation in the skin, since the *in vitro* octyl methoxycinnamate release mechanism is governed by its high lipophilicity and by the hydrophobicity and crystallinity of the polymeric material.

A comparison of Carbomer 940® hydrogels containing different nimesulide-loaded nanoparticles (nanocapsules, nanospheres, and nanoemulsion) was reported by Alves et al. [14]. All formulations

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presented pseudoplastic characteristics according to the Ostwald model and no thixotropic phenomenon was detected, regardless of the type of nanoparticles. In the following study, the penetration and distribution of nimesulide in human skin after the topical application of these formulations was investigated using the tape stripping technique and Franz-type diffusion cells [15]. Hydrogels containing nimesulide-loaded nanocapsules presented a higher penetration of nimesulide to the deeper skin layers in comparison with the formulations containing nimesulide-loaded nanospheres or nanoemulsion.

The efficiency of hair follicles to act as a drug reservoir after the topical application of a formulation containing nanoparticles was demonstrated by Ladermann et al. [18]. In this study, the authors observed deeper penetration of a dye into hair follicles after the application (with massage) of a formulation containing dye-loaded nanoparticles in comparison with one containing the dye in non-particle form. The differential stripping method revealed that nanoparticles were stored in the hair follicles for up to 10 days, while the nonparticle form could be detected only up to 4 days. In addition, Paese et al. [16] showed that the presence of polymeric nanocapsules in hydrogels did not produce contact sensitization in mice, demonstrating their low potential to cause contact allergic reactions.

Studies have also been conducted to develop semisolids containing CP-loaded nanoparticles. In this regard, Rao and Murthy [19] reported a lower absorption of the drug into the blood stream after topical application of HPMC gels containing CP-loaded liposomes compared to the same formulation containing the free drug. This result was attributed to higher drug accumulation in the skin, considering the results of *in vitro* studies on CP diffusion across rat skin membranes. Furthermore, Capó et al. [4] showed that the liposomal CP formulation presented a potency of 2.35 times higher compared to the formulation containing the free form. According to the authors, this increase could help to reduce the dosage of the drug, leading to a decrease in its adverse effects.

Kalariya et al. [5] demonstrated a lower mean flux value of CP from a cream containing drug-loaded solid lipid nanoparticles compared to a marketed cream containing the free drug. In addition, the nanostructured cream presented a better therapeutic response (1.9-fold higher for inflammation and 1.2-fold for itching) than the marketed formulation. Moreover, Senyigit et al. [20] showed that the incorporation of CP into lecithin/chitosan nanoparticles induced an accumulation of CP especially in the epidermis without any significant permeation across pig ear skin. Dilution of CP-loaded nanoparticles with chitosan gel (1:9) produced similar enhanced retention of CP in the epidermis and dermis compared to the commercial cream, even though the former contained ten times less CP. This is a remarkable finding in terms of reducing the side effects of CP.

Contact dermatitis is a frequent inflammatory skin disease that can be induced by exposure to low molecular weight chemicals, with both proinflammatory and antigenic properties [21]. This disease occurs in a delayed hypersensitivity reaction, mediated by cells through a mechanism that sensitizes the immune T lymphocyte to an antigen protein or a hapten linked to a protein [21]. Notable among the mediators able to modulate the actions of lymphocytes are adenine nucleosides and nucleotides, in particular, extracellular ATP, which is able to regulate the cell–cell interactions and is important in the processes of cell activation, differentiation, development, proliferation, and death, as well as effector lymphocyte response [22].

Extracellular nucleotides are messengers that modulate the exocrine and endocrine systems, the vasculature and hemostatic mechanisms, and musculoskeletal, immune and inflammatory cells [23]. These nucleotides represent an important means of modulating the activity of lymphocytes. The presence of an enzymatic mechanism is essential to maintaining the concentration of nucleotides in the extracellular space constant [22]. Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative, adenosine, are important signaling molecules that mediate diverse biological and pathological processes [24]. NTPDase (ecto-nucleoside triphosphate diphosphohydrolase; CD39) is an integral membrane protein that metabolizes extracellular ATP and ADP to AMP [25]. Kansas et al. [26] described the presence of CD39 on the surface of lymphocytes as well as endothelial cells. This enzyme has also been reported to be present on other leukocytes [27,28] as well as neoplastic cells in a number of hematologic malignancies [29–31]. CD39 has been demonstrated to have other effects in leukocytes including modulation of cytokine expression and the inflammatory response, as well as cell–cell adhesion [26,28,32]. It also affects the cell proliferation and apoptosis via its modulation of ATP levels in the pericellular fluid milieu [33]. The activity of NTPDase has been recognized as an activation marker necessary for effector lymphocyte function and participates in the processes of antigen recognition [22].

Recently, we reported the development of CP-loaded nanocarriers (nanocapsules, nanospheres, and nanoemulsion) as alternatives to topical administration of the drug [34]. All drug-loaded nanocarriers showed a controlled drug release and protection of the drug against UVA radiation. The polymer, poly(ε-caprolactone) (PCL), and the oil demonstrated an important influence on the drug release profile. Furthermore, PCL nanocapsules provided a better control over the drug released compared to other polyesters [35].

Taking all these considerations into account, the aim of this study was to develop a dermatological nanomedicine (hydrogel) containing CP-loaded polymeric nanocapsules and to evaluate its efficacy in the treatment of contact dermatitis, in order to obtain a new alternative for the treatment of skin disorders. Hydrogels containing CP-loaded nanoemulsions were also prepared in order to evaluate the effect of the polymeric layer on their physicochemical and rheological properties. Our main hypothesis was that by controlling the clobetasol propionate release from hydrogels through its association with nanocarriers, it may be possible to reduce the total dose of drug to be administered in a cutaneous disorder-like contact dermatitis. The *in vivo* protocol was based on the induction of contact dermatitis in rats using a dispersion of nickel sulfate in solid Vaseline at 5%.

2. Materials and methods

2.1. Materials

The CP used in this study was donated by Neo Quimica (Goiás, Brazil). The PCL and sorbitan monostearate were acquired from Sigma–Aldrich (São Paulo, Brazil). The caprylic/capric triglyceride mixture and imidazolidinyl urea were supplied by Brasquim (Porto Alegre, Brazil) and Alpha Quimica (São Paulo, Brazil), respectively, and polysorbate 80 and polyethylene glycol 400 by Henrifarma (São Paulo, Brazil). Acetate cellulose membranes (0.45 μm pore size) were acquired from Millipore (São Paulo, Brazil). Carbomer Ultrez® 10 NF and triethanolamine were acquired from DEG (São Paulo, Brazil). Clobetasol propionate commercial gel (Clob-X 0.05%, batch 2880038, Galderma) was purchased locally. HPLC grade methanol was acquired from Tedia (São Paulo, Brazil). Ethanol and acetone were acquired from Impex (São Paulo, Brazil). Nucleotides, sodium azide, HEPES, and Trizma base were purchased from Sigma Aldrich (St. Louis, MO, USA). Ficoll-Hypaque (Lymphoprep™) was purchased from Nycomed Pharma (Oslo, Norway), and all other reagents used in the experiments were of analytical grade and of the highest purity. All chemicals and solvents were used as received.

2.2. Preparation and characterization of nanocapsules and nanoemulsion

CP-loaded lipid-core nanocapsules (CP-NC) and nanoemulsion (CP-NE) were prepared by interfacial deposition of the preformed polymer [36] and spontaneous emulsification [37], respectively, at a drug concentration of 0.5 mg mL^{-1}, according to the quali-quantitative formula described by Fontana et al. [34]. To prepare the CP-NC, an organic solution containing the drug, a medium-chain triglyceride mixture, sorbitan monostearate, PCL, and acetone was poured under moderate magnetic stirring into an aqueous solution containing polysorbate 80. The magnetic stirring was maintained for 10 min. The acetone was then removed and the aqueous phase concentrated by evaporation (bath at 40 °C) under reduced pressure. The CP-NE was prepared using the same procedure, omitting the addition of the polymer in the organic phase. Blank nanocapsules and nanoemulsions were prepared without the addition of the drug into the organic phase. All formulations were prepared under protection from light and kept in the dark throughout the experiment. Particle sizes and polydispersity indices of the colloidal dispersions were estimated by photon correlation spectroscopy (PCS) after adequate dilution of an aliquot of the suspension in purified water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK). Zeta potentials were measured using the same instrument at 25 °C, after dilution of the samples in 10 mM NaCl aqueous solution. Drug content (mg mL^{-1}) was determined after dissolution of nanoparticles in acetonitrile. CP was assayed by liquid chromatography according to a previously validated method [38].

2.3. Preparation of hydrogels

Three batches of each hydrogel were prepared with a mortar and pestle, according to the quali-quantitative formula described in Table 1. Carbomer Ultrez® 10 NF (acrylic acid polymer) was used for the preparation of the hydrogels due to its wide use in pharmaceutical formulations and its fast redispersion in water [39]. Briefly, Carbomer Ultrez® 10 NF was dispersed using the CP-loaded nanoparticle formulations (NC or NE) resulting in a concentration of 0.05% of the drug. This concentration was chosen considering the formulation available in the market containing clobetasol propionate for dermal use (0.05%). This dispersion was neutralized with triethanolamine to obtain an adequate semisolid formulation for cutaneous application. Imidazolidinyl urea was added as a preservative. Using the same method, formulations containing blank nanoparticles were prepared together with a control hydrogel prepared without the addition of any nanoparticle formulation. In addition, a hydrogel containing nonencapsulated (free) CP was prepared. In this case, water and an ethanolic solution containing the drug were used instead of the nanoparticle formulation during the dispersion of the polymer. The formulations are referred to herein as: HG-CP-NC and HG-CP-NE, for the hydrogels containing CP-loaded nanocapsules and nanoemulsion, respectively; HG-B-NC and HG-B-NE for the respective hydrogels containing blank nanoparticles; HG-CP for the hydrogel containing nonencapsulated CP; HG-B1 (without drug and ethanol – placebo hydrogel 1) and HG-B2 (without drug – placebo hydrogel 2) for control hydrogels.

2.4. Physicochemical characterization of hydrogels

Hydrogels were characterized according to the following characteristics: drug content, pH, presence of intact nanoparticles in the hydrogel, spreadability, rheological behavior, and *in vitro* drug release. In order to establish an adequate comparison, a commercial hydrogel (HG-C) was characterized in parallel (CP commercial gel – Clob-X 0.05%, batch 2880038, Galderma, São Paulo, Brazil).

2.4.1. Assay of CP in hydrogels

The content of CP in the hydrogels was assayed by liquid chromatography (LC). Approximately, 1.0 g of each formulation was placed in a 25 mL volumetric flask. Methanol was added, and the flask was maintained under moderate stirring by ultrasound for 30 min. This sample was centrifuged, filtered through a paper filter (Quantitative filter, JP41 – 28 μm), and through a 0.45-μm nylon membrane before LC analysis. The chromatographic system was validated and consisted of a Gemini RP-18 column (250 × 4.60 mm, 5 μm particle size, 110 Å pore diameter, Phenomenex, Torrance, USA) and a Shimadzu LC-20A system, (LC-20AT pump, SPD-M20A photodiode-array (PDA) detector, CBM-20A system controller, SIL-20A auto-sampler, Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 1.0 mL min^{-1} consisted of methanol-water (80:20 v/v). The volume injected was 20 μL, and the drug was detected at 241 nm. The method was linear ($r = 0.9996 \pm 0.0001$, $y = 39220.89x - 9236.51$) in the range of 5.0–40.0 μg mL^{-1}, precise (repeatability: 0.05–0.25%; intermediate precision: 0.29–0.96%), accurate (100.12%) and specific. The specificity was tested in the presence of the excipients of the formulations (nanoparticles and hydrogels) and demonstrated that these components did not alter the CP assay. Peak-purity evaluation using the PDA detector showed that no impurities and/or excipients were co-eluting with the CP peak.

2.4.2. Determination of pH

The pH values of the hydrogels were determined in the dispersion of an aliquot of each formulation in ultrapure water (10%, w/v), using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil).

2.4.3. Analysis of intact nanoparticles in the hydrogel

The presence of nanoparticles in the hydrogel was analyzed by photon correlation spectroscopy (PCS) (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK). Before the analysis, an aliquot of hydrogel was diluted in purified water (500×) and shaken (vortex) until its complete redispersion. Analyses were made in triplicate [6].

Table 1
Quali-quantitative composition of hydrogels.

Component	HG-CP-NC or NE	HG-B-NC or NE	HG-B1	HG-CP	HG-B2
Carbopol Ultrez® 10 NF	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
Imidazolidinyl urea	0.6 g	0.6 g	0.6 g	0.6 g	0.6 g
Triethanolamine	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Clobetasol propionate	–	–	–	0.05 g	–
CP-NC or CP-NE	ad 100 g	–	–	–	–
B-NC or B-NE	–	ad 100 g	–	–	–
Ethanol	–	–	–	ad 50 g	ad 50 g
Ultra pure water	–	–	ad 100 g	ad 50 g	ad 50 g

2.4.4. Evaluation of rheological properties of hydrogels

The rheological study was carried out at 25 ± 1 °C using a rotational viscosimeter (LVDV II + Pro model, Brookfield, USA) spindle SC4-25 with a small sample adaptor. The data obtained were analyzed with the Rheocalc software (V3.1-1 version, Brookfield, USA). The shear stress ramp was applied for 1200 s, and 20 different points were recorded, using a shear rate interval of 0.05 s^{-1}. The rheograms were analyzed using different flow models: Bingham ($\tau = \tau + \eta\gamma$), Casson ($\tau = \tau_o^{0.5} + \eta^{0.5}\gamma^{0.5}$), Ostwald ($\tau = \kappa\gamma^{0.5}$) and Herschel–Bulkley ($\tau = \tau_o + \kappa\gamma^{0.5}$), where τ is the shear stress, τ_o is the yield stress, η is the viscosity, n is the flow index, κ is the consistency index, and γ is the shear rate [40].

2.4.5. Determination of the spreadability

The evaluation of spreadability was performed at 25 ± 1 °C and using the parallel plate method with weights previously described by Borghetti and Knorst [41]. The sample was introduced in the central hole (1 cm) of a glass plate mold. The plate mold was carefully removed, and the sample was pressed between with plates of known weights, with intervals of 1 min between each plate. The spreading area reached by samples between each addition of a glass plate was measured in millimeters along the vertical and horizontal axes. Results were expressed in terms of the spreading area as a function of the applied mass according to the following equation (Eq. (1)) and represent the mean of three determinations:

$$S_i = \frac{d^2 \cdot \pi}{4} \quad (1)$$

in which S_i is the spreading area (mm^2) after the application of a certain mass i (g), and d is the mean diameter (mm) reached by each sample. The spreading area was plotted against the plate weights to obtain the spreading profiles.

The spreadability factor (S_f) was also calculated and represents the spread of a formulation on a smooth horizontal surface when a gram of weight is placed on top of it, under the conditions described in the methodology previously. The following equation (Eq. (2)) is used to calculate the spreadability factor [6]:

$$S_f = \frac{A}{W} \quad (2)$$

in which S_f (mm^2 g) is the spreadability factor resulting from the ratio between (A) the maximum spread area (mm^2) after the addition of the sequence of weights used in the experiment and (W) the total weight added (g).

2.5. In vitro drug release assay from hydrogels

The *in vitro* release of CP from HG-CP-NC, HG-CP-NE, and HG-CP was studied using vertical Franz diffusion cells at 37 ± 0.5 °C ($n = 6$). This study aimed to evaluate whether the nanoencapsulation of CP affects the nature of its release from the hydrogels. Two independent experiments ($n = 3$ for each independent experiment) were carried out for each formulation. An acetate cellulose membrane (0.45 μm pore size) was fitted between the donor and receptor compartments. The diffusion area was 2.14 cm^2, and the receptor chamber volume was 6.0 mL. The receptor medium consisted of water/Tween 80®/PEG 400 (60:0.5:40 v/v) [34,35,42] and was continuously stirred. According to our previous studies, it was necessary to add Tween 80® and PEG 400 at the concentration above in order to maintain the sink conditions during all experiment [34]. One gram of hydrogel containing 500 μg of CP (infinite dose) was evenly spread on the membrane surface. Half a milliliter of the receptor medium was taken at predetermined time intervals (2, 3, 4, 5, 6, 7 and 8 h) and replaced by an equal volume of fresh medium. The amount of CP released was determined by LC according to the experimental conditions described in Section 2.4.1., excepting the volume of injection, which was adjusted to 100 μL in order to allow the quantification of lower amounts of CP. These conditions were previously validated for *in vitro* drug release studies [34].

The Higuchi model ($C = k \cdot t^{0.5}$) was used to evaluate the influence of the nanoparticle structure on the profiles for the drug release from the hydrogels. C is the cumulative amount of drug released at time t, and k is a constant reflecting the design variables of the system related to the diffusion area, diffusion coefficient, and drug solubility in the system [43,44]. The mathematical modeling was performed using the software MicroMath® Scientist® for Windows™.

2.6. Evaluation of the in vivo efficacy

2.6.1. Animals

Female Wistar rats were separated into five groups of eight animals. The animals, 90 days old and weighing 260–280 g, were maintained in a standard dark–light cycle at room temperature. The rats had free access to food (standard laboratory rat chow) and water. The animal experiment was performed according to the official governmental guidelines in compliance with the Brazilian Association for Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal) and with the approval of the ethics committee (Reg. no. 23081.014716/2009-83) of the Federal University of Santa Maria, Brazil.

2.6.2. Dermatitis induction by 5% nickel sulfate

Contact dermatitis was induced by 5% nickel sulfate in solid Vaseline according to the protocol described by Brum et al. [45]. Animals were divided into five groups ($n = 8$). After tricotomization, all groups received sensitization with nickel sulfate in the abdomen, except the first group which received only solid Vaseline and remained under the same environmental and feeding conditions as the other groups, this being the control group (C).

The induction of dermatitis was performed 6 days after sensitization by the application of 5% nickel sulfate in solid Vaseline (5 applications with intervals of 72 h) in each ear after tricotomization. The first group received only solid Vaseline and these rats were euthanized 72 h after the last application of the sensitization agent. The second group was induced to allergic contact dermatitis, which was not managed, and the rats were euthanized 72 h after the last application of nickel sulfate, being the positive control (D). The third group received the topical administration in each ear of the hydrogel containing blank nanocapsules (D-HG-B-NC), the fourth and fifth groups received topical administration in each ear of the hydrogel containing free clobetasol or clobetasol-loaded nanocapsules (D-HG-CP and D-HC-CP-NC, respectively). Group 4 was treated daily with 2 mg of the respective formulation (corresponding to 1 μg of clobetasol propionate) for 5 days. Group 5 received the same amount of the respective formulation, on days 1, 3, and 5. All hydrogels were applied uniformly throughout the ear tissue with massage in order to obtain a better drug penetration [18]. After the completion of each treatment, the animals were euthanized and the blood was collected by cardiac puncture to determine the NTPDase activity.

2.6.3. NTPDase activity of lymphocytes

Mononuclear leukocytes were isolated from rat blood collected with EDTA and separated using Ficoll-Hypaque density gradients as described by Böyum [46]. After the isolation of mononuclear cells, NTPDase activity was determined by colorimetric assay in compliance with Leal et al. [47]. All samples were run in duplicate or triplicate, and specific activity is reported as nmol Pi released/min/mg protein. Protein was measured by the Coomassie Blue

method using bovine serum albumin as the standard as described by Bradford [48].

2.7. Statistical analysis

All formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). One-way analysis of variance (ANOVA) was used to compare the experimental data. Post hoc multiple comparisons were carried out using Tukey's test for significance considering p-values $\leqslant 0.05$. The analysis was performed using the SigmaStat statistical program (Version 3.0, Jandel Scientific, USA). To determine the differences between the treatments in terms of the NTPDase activity of lymphocytes, the data were subjected to ANOVA (followed by Tukey–Kramer or Kruskal–Wallis Tests) considering significance at p-values $\leqslant 0.05$.

3. Results and discussion

3.1. Characterization of nanoparticles

CP-NC and CP-NE presented, macroscopically, a homogeneous appearance, and the drug content was close to their theoretical value of 0.5 mg mL^{-1} (0.510 ± 0.006 mg mL^{-1} and 0.502 ± 0.008 mg mL^{-1}, respectively). All formulations had neutral pH values and nanometric mean size (between 180 and 220 nm), a low polydispersity index (<0.25) and negative zeta potential, according to our previous study on CP-loaded nanoparticles [34]. These characteristics were checked as a form of quality control before the use of each formulation in the preparation of the hydrogels.

3.2. Characterization of hydrogels

The use of CP-loaded nanoparticles to prepare the hydrogels allowed these semisolid dosage forms to be obtained without the use of nonaqueous cosolvents. Marketed formulations currently available are prepared using polyethylene glycol 200 as a cosolvent. Carbomer Ultrez® 10 NF hydrogels were prepared using freshly prepared CP-NC and CP-NE (0.5 mg mL^{-1}), which are referred to herein as HG-CP-NC and HG-CP-NE, respectively. For comparison purposes, hydrogels containing nonencapsulated CP (HG-CP), blank nanoparticles (HG-B-NC and HG-B-NE), and placebo formulations (HG-B1 and HG-B2) were also prepared. All hydrogels containing nanoparticles showed white color, glossy, and homogeneous aspect, and satisfactory organoleptic characteristics, regardless of the nanostructure. On the other hand, HG-B1 and HG-B2 as well as HG-CP presented a transparent and homogeneous aspect.

Table 2 shows the characteristics of the hydrogels containing drug-loaded nanoparticles, the hydrogel containing the nonencapsulated drug (HG-CP) as well as the placebo hydrogels (HG-B1, HG-B2). All hydrogels had drug contents close to their theoretical value (0.50 mg g^{-1}) and pH values of around 6.0, which is suitable for the topical application [15]. Formulations prepared with blank nanoparticles presented similar pH values compared to those containing the drug (5.83 ± 0.16 and 5.92 ± 0.14 for HG-B-NC and HG-B-NE, respectively). Neither the nanoparticles nor the presence of the drug appeared to influence this parameter ($p > 0.05$). However, statistical analysis showed lower pH values for all formulations compared to the commercial formulation (7.20 ± 0.05).

Table 2
Physicochemical characteristics of hydrogels (mean ± standard deviation, $n = 3$).

Formulation	Drug content (mg mL^{-1})	pH
HG-CP-NC	0.518 ± 0.006	5.97 ± 0.19^{a,b}
HG-CP-NE	0.519 ± 0.009	5.70 ± 0.14^{a}
HG-B1	–	6.16 ± 0.06^{b}
HG-CP	0.509 ± 0.011	6.11 ± 0.08^{b}
HG-B2	–	5.67 ± 0.02^{a}

Means, in column, with the same letter are not statistically different (ANOVA, Tukey test, $p \leqslant 0.05$).

Table 3
Mean particle size and polydispersity index (PDI) of CP-NC and CP-NE dispersions and their respective hydrogels ($n = 3$).

Formulation	Particle sizes (nm)	PDI
CP-NC	213 ± 2	0.14 ± 0.01
CP-NE	205 ± 1	0.14 ± 0.03
HG-CP-NC	206 ± 1	0.10 ± 0.02
HG-CP-NE	190 ± 3	0.10 ± 0.01

In order to verify the presence of intact nanoparticles in the hydrogels, the particle size was analyzed by PCS after aqueous redispersion (Table 3). Analysis of both formulations containing the nanoencapsulated drug (HG-CP-NC and HG-CP-NE) showed the presence of nanometric particles. This result is in agreement with previous reports verifying the presence of intact nanoparticles in Carbomer hydrogels by freeze-fracture scanning electron microscopy (SEM) [12] and transmission electron microscopy (TEM) [6].

The rheological properties of the semisolid forms are also of fundamental importance, in order to predict the effects of the formulations and improve their quality and stability [41]. Fig. 1 shows the rheograms of formulations containing CP (Fig. 1A) and placebo formulations (Fig. 1B) obtained by plotting the applied shear rate as a function of the shear stress. As can be seen in the rheograms,

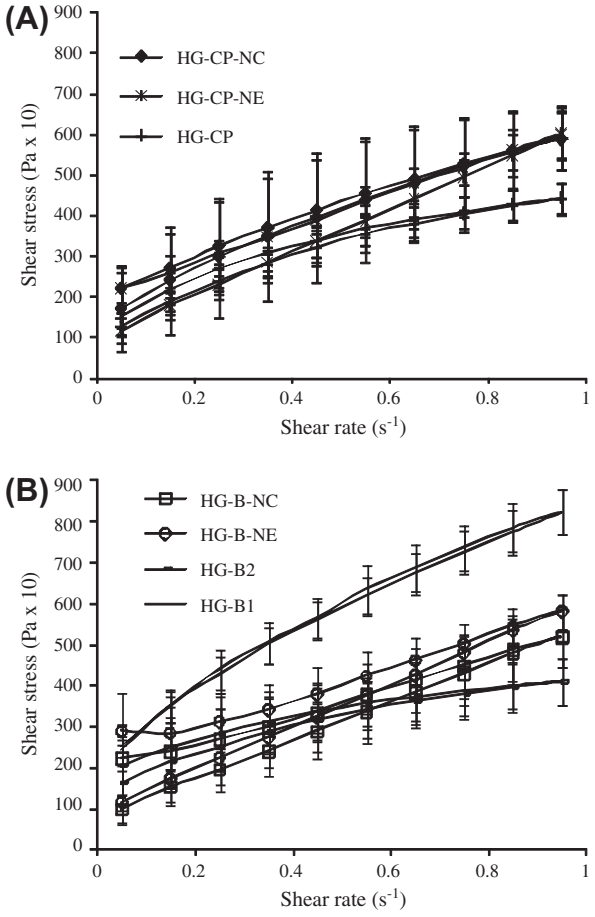

Fig. 1. Rheograms of hydrogels ($n = 3$): (A) hydrogels containing CP; (B) blank hydrogels (placebo hydrogels).

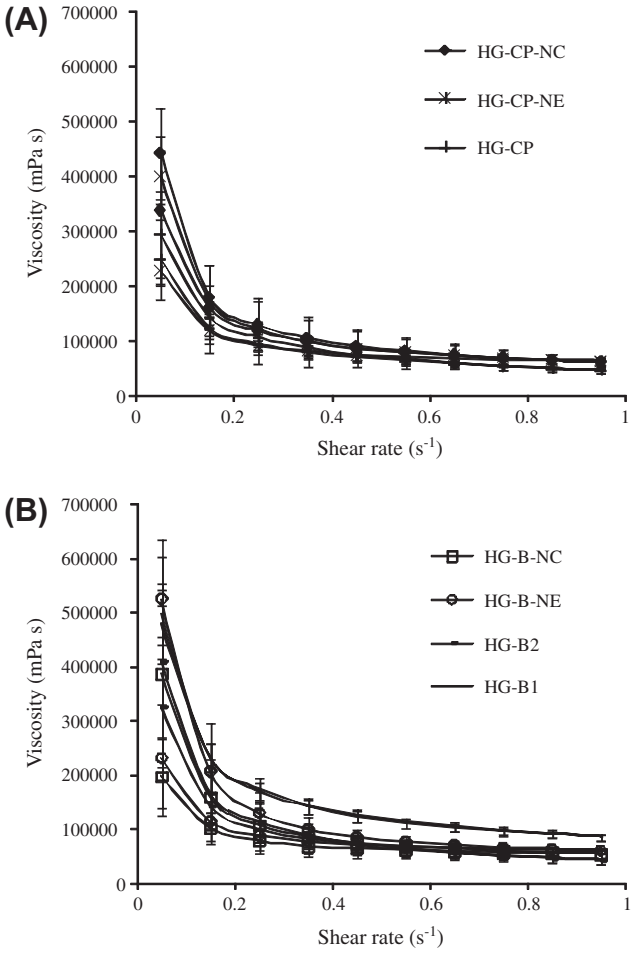

Fig. 2. Graphic representation of viscosity (mPa s) of hydrogels in relation to the shear rate (s^{-1}) ($n = 3$): (A) hydrogels containing CP; (B) blank hydrogels (placebo hydrogels).

the formulations showed non-Newtonian behavior, in which the viscosity decreases with increasing shear rate [40], as better observed in Fig. 2A and B. This phenomenon indicates the breakdown of the three-dimensional structure of the system and is of particular interest in the area of semisolid technology, since it makes the system more fluid when submitted to external pressure, and therefore, it spreads more easily in the region to which the pressure is applied [49]. Alves et al. [14] also observed that the non-Newtonian behavior of gels is not influenced by the presence of nanocapsules, nanospheres, and nanoemulsion.

There are several models which may be used to establish the flow index (n) and to obtain a better understanding of the rheological behavior of different non-Newtonian systems, such as the Bingham, Casson, Ostwald, and Herschel–Bulkley models. The rheograms obtained for all formulations showed that the Herschel–Bulkley model gave the best fit, providing regression coefficients higher than 0.99 (Table 4). For topical anti-inflammatory formulations, the consistency of the samples is a particularly important feature, due to the fact that they must be applied to the skin in thin layers [50]. The yield stress (YS), flow index (n), and consistency index (κ) were determined for all formulations after preparation, and the results are shown in Table 5. According to the selected model, almost all the flow indices were lower than 1 indicating a pseudoplastic behavior. However, the flow index of the formulation containing the drug-loaded nanoemulsion (HG-CP-NE) was close to 1, with a YS of 8.66 ± 0.84 (Pa), demonstrating a tendency toward plastic behavior [49]. No significant difference was observed between the formulations containing the drug-loaded nanosystems and their respective placebo formulations ($n = 0.99 \pm 0.13$ and 1.00 ± 0.06 for HG-B-NC and HG-B-NE, respectively) and control formulation (HG-B1), indicating that the presence of the drug did not affect this parameter (ANOVA, $p > 0.05$). In addition, HG-CP prepared with a hydroalcoholic solution and its respective control formulation (HG-B2) also presented flow indices below 1, as did the commercial product ($n = 0.35 \pm 0.27$), indicating pseudoplastic behavior. Pseudoplastic and plastic behaviors have been previously reported for Carbomer hydrogels containing polymeric nanoparticles and nanoemulsions. Alves et al. [14] and Paese et al. [16] reported pseudoplastic behavior for hydrogels containing nimesulide-loaded nanoparticles and benzophenone-loaded nanocapsules. In this case, the rheograms reportedly gave the best fit to the Ostwald model. Milão et al. [12] reported a plastic behavior for hydrogels containing diclofenac-loaded nanocapsules, with a good fit being obtained for the Casson model. These different findings could be related to the differences in the type of the polymer (Carbomer 940 × Carbomer Ultrez NF 10) and its concentration in the formulation (0.2 × 0.5%). Carbomer 940 disperses in water to form acidic colloidal dispersions of low viscosity which when neutralized produce highly viscous gels. On the other hand, Carbomer Ultrez 10 is a carbomer resins that wets quickly, yet hydrates slowly while possessing a lower unneutralized dispersion viscosity [51].

Regarding the consistency index, all hydrogels containing drug-loaded nanoparticles as well as nonencapsulated CP showed similar values (ANOVA, $p > 0.05$). No difference was observed in relation to their placebo formulations ($\kappa = 46{,}940 \pm 5718$ mPa s and $51{,}419 \pm 3237$ mPa s for HG-B-NC and HG-B-NE, respectively). In addition, all formulations presented lower consistency indices in comparison with the commercial formulation ($187{,}534 \pm 53{,}690$ mPa s).

The pseudoplastic behavior is characterized by the YS, which must be exceeded before the fluid deforms or flows [49]. According to our results (Table 5), small yields were required for the formulations to start to flow and no statistical differences were observed between the formulations containing the different drug-loaded nanoparticles (ANOVA, $p > 0.05$). In addition, no significant difference was observed between the formulations containing the drug-loaded nanosystem and their respective placebo formulations (YS = 7.49 ± 1.83 Pa and 9.22 ± 1.21 Pa for HG-B-NC and HG-B-NE,

Table 4
Regression coefficient (r^2) for various flow models in shear rate–shear stress curve.

Formulation	Binghan	Casson	Ostwald	Herschel–Bulkley
HG-CP-NC	0.966 ± 0.028	0.982 ± 0.002	0.942 ± 0.037	0.998 ± 0.001
HG-CP-NE	0.987 ± 0.003	0.988 ± 0.002	0.935 ± 0.004	1.000 ± 0.001
HG-B1	0.955 ± 0.001	0.992 ± 0.002	0.966 ± 0.001	0.999 ± 0.001
HG-CP	0.880 ± 0.006	0.961 ± 0.002	0.958 ± 0.006	0.993 ± 0.003
HG-B2	0.955 ± 0.023	0.989 ± 0.008	0.991 ± 0.012	1.000 ± 0.005

Table 5
Flow index (n), consistency index (κ), yield stress (YS), and spreadability factor (S_f) of the hydrogels.

Formulation	n	κ (mPa s)	YS (Pa)	S_f ($mm^2\ g^{-1}$)
HG-CP-NC	$0.81 \pm 0.39^{a,b}$	$53{,}479 \pm 10400^{a}$	5.32 ± 3.67^{a}	3.87 ± 0.34^{a}
HG-CP-NE	0.96 ± 0.03^{a}	$53{,}732 \pm 5111^{a}$	8.66 ± 0.84^{a}	2.64 ± 0.18^{b}
HG-B1	$0.63 \pm 0.09^{a,b}$	$71{,}830 \pm 7531^{a}$	13.20 ± 10.50^{a}	3.13 ± 0.37^{b}
HG-CP	0.49 ± 0.05^{b}	$44{,}463 \pm 3330^{a}$	1.52 ± 1.35^{a}	3.69 ± 0.07^{a}
HG-B2	0.41 ± 0.06^{b}	$35{,}667 \pm 2741^{a}$	6.60 ± 6.76^{a}	3.53 ± 0.25^{a}

Means, in column, with the same letter are not statistically different (ANOVA, Tukey test, $p \leqslant 0.05$).

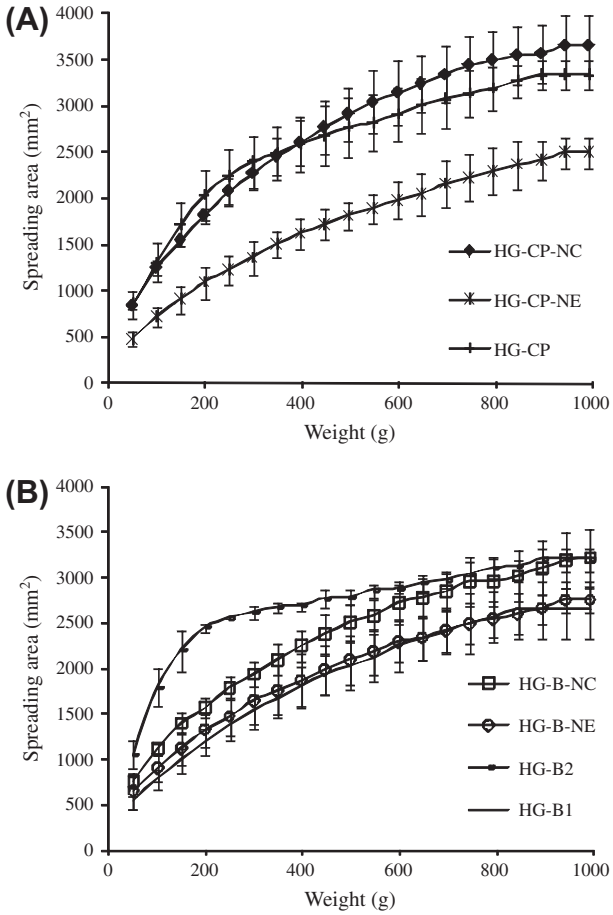

Fig. 3. Graphic representation of spreadability of hydrogels ($n = 3$): (A) hydrogels containing CP; (B) blank hydrogels (placebo hydrogels).

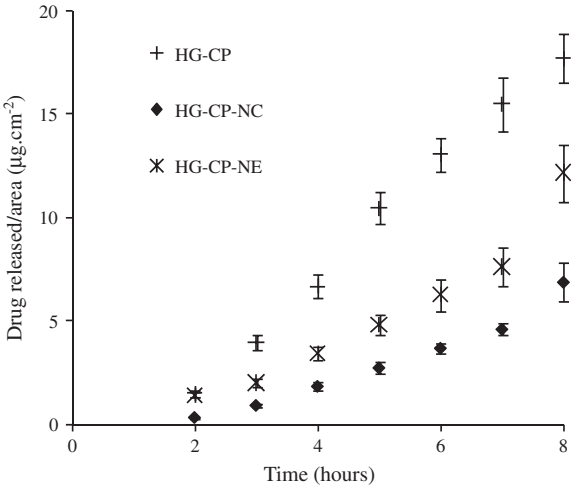

Fig. 4. Release profile of clobetasol propionate from hydrogel containing CP-loaded NC, CP-loaded NE, and hydrogel containing free CP using vertical Franz diffusion cells ($n = 6$).

respectively). Similarly, no significant difference was observed between the formulation containing the nonencapsulated drug (HG-CP) and its respective control formulation (HG-B2). These results demonstrate that the presence of the drug or the polymeric layer does not show any influence on this parameter (ANOVA, $p > 0.05$). Furthermore, the commercial hydrogel showed a similar YS (YS = 4.46 ± 14.26 Pa) to those of all formulations (ANOVA, $p > 0.05$).

Spreadability is another important characteristic of dermatological medicines to be evaluated during developmental studies, this being responsible for the correct dosage transfer to the target site and easy application on the substrate [52]. The results for the spreadability of the hydrogels as a function of the added weight are shown in Fig. 3, which shows that the incorporation of the nanoparticles in the semisolid did not modify their profiles compared to HG-CP and HG-B1. In addition, hydrogels containing polymeric nanoparticles (NC) showed a tendency toward better spreadability (higher spread area as a function of weight) compared to the hydrogel containing NE, regardless of the presence of the drug (Fig. 3A and B).

The spreadability factor (Table 5) was calculated to analyze the differences between the formulations. The formulation containing drug-loaded nanoparticles (HG-CP-NC) showed the highest spreadability factor. This value was similar to those obtained for HG-CP and HG-B2 (ANOVA, $p \leqslant 0.05$). The lowest spreadability factor was obtained for the HG-CP-NE. In addition, similar results were obtained for formulations containing blank nanoparticles compared to their respective drug-loaded formulations ($S_f = 3.48 \pm 0.36$ mm^2 g^{-1} and 2.91 ± 0.15 mm^2 g^{-1} for HG-B-NC and HG-B-NE, respectively). These results are in agreement with the spreadability behavior observed in Fig. 3. However, HG-C showed a higher spreadability factor ($S_f = 5.12 \pm 0.31$ mm^2 g^{-1}) ($p \leqslant 0.05$) compared to all other hydrogels, which may be attributed to the presence of some excipients in its composition, such as alantoin and poly(ethyleneglycol). Alves et al. [14] evaluated the spreadability of hydrogels using the same method and did not detect any significant difference between formulations containing nanoparticles (NC, NS and NE) and the gel control (Carbomer 940® at 0.2% w/w).

3.3. In vitro CP release from hydrogels

Studies on the *in vitro* drug release from the hydrogels containing CP-loaded nanoparticles (HG-CP-NC and HG-CP-NE) and the hydrogel containing nonencapsulated clobetasol propionate (HG-CP) were carried out using vertical diffusion Franz cells. Fig. 4 shows the *in vitro* CP release profiles for the different hydrogels. HG-CP showed a higher amount of CP released after 8 h compared to the formulations containing the drug-loaded nanoparticles. Among these formulations, HG-CP-NC presented a lower amount of drug released per cm^2 after 8 h compared to HG-CP-NE (ANOVA, $p \leqslant 0.05$).

The drug flux of each formulation was determined by the slope of the curve obtained by plotting the amount of CP released per cm^2 against the square root of time [53]. Hydrogels containing the nanoencapsulated drug showed a lower release rate of CP (HG-CP-NC: 1.03 ± 0.11 μg cm^{-2} h and HG-CP-NE: 1.65 ± 0.19 μg cm^{-2} h) compared to the hydrogels containing the nonencapsulated drug (HG-CP: 2.79 ± 0.22 μg cm^{-2} h) (ANOVA, $p \leqslant 0.05$). Regarding the formulations containing the nanostructured systems, HG-CP-NC presented a lower release rate compared to the HG-CP-NE ($p \leqslant 0.05$). In addition, the results were fitted using the Higuchi mode and presented a good correlation coefficient and model selection criteria. The Higuchi constants (k) are shown in Table 6. The results of the *in vitro* drug release studies demonstrated the importance of the presence of the polymer in relation to controlling the *in vitro* release of CP from hydrogels. The influence of the viscosity can be refuted. All drug-loaded nanoparticle hydrogels presented similar consistency indices (Table 5), as previously discussed. Furthermore, the importance of the polymeric layer in nanocapsules, compared to nanoemulsions, in controlling

Table 6
Observed rate constants (k), correlation coefficients (r), and MSC obtained by fitting the clobetasol propionate release from free clobetasol propionate (HG-CP) and from different nanocarriers (HG-CP-NC, HG-CP-NE) according to the Higuchi's square root model.

Higuchi model	HG-CP	HG-CP-NC	HG-CP-NE
k (h^{-1})	4.822 ± 0.308[a]	1.497 ± 0.122[c]	2.657 ± 0.258[b]
r (range)	0.994 ± 0.003	0.959 ± 0.017	0.932 ± 0.026
MSC (range)	0.692 ± 0.047	0.430 ± 0.096	0.466 ± 0.080

Means, in line, with the same letter (a, b and/or c) are not statistically different (ANOVA, Tukey test, $p \leqslant 0.05$).

the release of this drug has been previously reported for the respective nanoparticle liquid dispersions [34].

3.4. NTPDase activity of lymphocytes in contact dermatitis

The *in vivo* NTPDase activity of lymphocytes after each treatment is shown in Fig. 5. A significant increase in NTPDase activity was observed in lymphocytes of the group treated with hydrogel containing CP-loaded nanocapsules (D-HG-CP-NC), in relation to ATP and ADP (Fig. 5A and B, respectively), compared to all other groups ($p < 0.01$). It should be highlighted that this difference could be observed despite the lower frequency and number of the treatments with this formulation. The ADP and the ATP hydrolysis values for the group treated with hydrogel containing nonencapsulated CP and the control groups were not statistically different.

The higher NTPDase activity may be associated with the high levels of extracellular ATP resulting from the inflammatory process, which occurs in cases of allergic contact dermatitis. During the dermatitis, these high levels of ATP would have an affinity for P2X7 purinergic receptors, leading to a Th1 pattern of immune response with the production of inflammatory cytokines. The NTPDase would act by decreasing the levels of ATP, which in low concentration would bind to the P2Y receptors, reversing the pattern of immune response to Th2 with the release of anti-inflammatory cytokines. Thus, it is possible that the increased hydrolysis of adenine nucleotides also leads to an increase in the extracellular adenosine concentration, which has immunosuppressive and anti-inflammatory effects. Adenosine plays a central and direct role in the regulation of inflammatory responses and in limiting inflammatory tissue destruction [24,54].

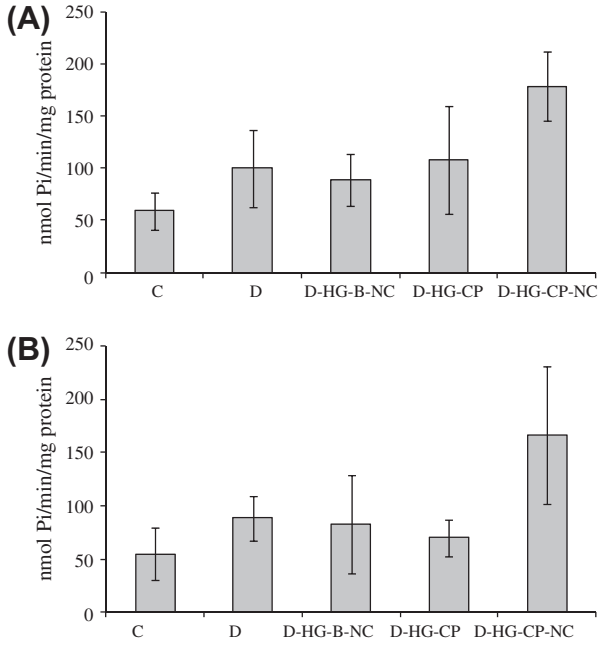

Fig. 5. ATP (6A) and ADP (6B) hydrolysis in lymphocytes obtained from the control group (C), contact dermatitis group (D), groups with dermatitis treated with HG-B-NC or HG-CP or HG-CP-NC. Data were analyzed statistically by one-way ANOVA followed by the Tukey–Kramer test (6A) and Kruskal–Wallis Test (6B), $p < 0.05$.

In this context, the higher NTPDase activity in the treatment with the hydrogel containing CP-loaded nanocapsules, at intervals of 48 h, could be related to the higher anti-inflammatory effect in comparison with nonencapsulated clobetasol propionate. Therefore, the best result observed for the hydrogel containing CP-loaded nanocapsules at 0.05%, even after a longer interval of time, may be related to the slower CP release as shown in Fig. 5 and its accumulation in the hair follicles as previously demonstrated by Lademann et al. [18] for substances in the form of nanoparticles. In addition, the small particle size of the nanocarriers ensures close contact with the stratum corneum [17]. As a final point, the similar behavior of the groups with and without treatment with the hydrogel containing blank nanocapsules should be noted. This result demonstrates the safe application of the nanostructured formulation developed, which did not appear to stimulate an inflammatory or immune response using the contact dermatitis model. This result represents an important contribution to nanotoxicological studies of polymeric nanoparticles and verifies the possibility for their safe use in topical administration.

4. Conclusions

Hydrogels containing CP-loaded nanoparticles were developed without using nonaqueous cosolvents, showing adequate drug content, pH compatible with topical application, and pseudoplastic thixotropic behavior. *In vitro* drug release studies showed a better controlled CP release, following the Higuchi model, from hydrogels containing the nanoencapsulated drug, compared to the hydrogels containing the nonencapsulated drug. The best release control was obtained for semisolid formulations containing CP-NC, showing the influence of the presence of the polymer in obtaining this behavior. The hydrogel containing 0.05% CP-NC showed higher NTPDase activity in the treatment of contact dermatitis and a greater immunosuppressive effect compared to the hydrogel containing the nonencapsulated form. The nanoencapsulation of clobetasol propionate led to a better control of the drug release from the semisolid nanomedicine, providing better *in vivo* dermatological efficacy. Evaluation of the effects of administering hydrogels containing blank nanoparticles contributes to the safe use of this kind of formulation by cutaneous administration. This formulation can be recommended as a dermatological nanomedicine and is potentially a more efficient treatment for contact dermatitis.

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References

[1] T. Senyigit, C. Padula, Ö. Özer, P. Santi, Different approaches for improving skin accumulation of topical corticosteroids, Int. J. Pharm. 380 (2009) 155–160.
[2] U.R. Hengge, T. Ruzicka, R.A. Schwartz, M.J. Cork, Adverse effects of topical glucocorticosteroids, J. Am. Acad. Derm. 54 (2006) 1–15.
[3] N.N. Zöller, S. Kippenberger, D. Thaçi, K. Mewes, M. Spiegel, A. Sättler, D. Schultz, J. Bereiter-Hahn, R. Kaufmann, A. Bernd, Evaluation of beneficial and adverse effects of glucocorticoids on a newly developed full-thickness skin model, Toxicol. In Vitro 22 (2008) 747–759.
[4] J.I.T. Capó, X.P. Gutiérrez, C.C. Dominguez, Incremento de la actividad timolítica del clobetasol em forma liposomal, Rev. Cubana Farm. 38 (2004) 2.

[5] M. Kalariya, B.K. Padhi, M. Chougule, A. Misra, Clobetasol propionate solid lipid nanoparticles cream for effective treatment if eczema: formulation and clinical implications, Indian J. Exp. Biol. 43 (2005) 233–240.
[6] M.L. Marchiori, G. Lubini, G. Dalla Nora, R.B. Friedrich, M.C. Fontana, A.F. Ourique, M.O. Bastos, L.A. Rigo, C.B. Silva, S.B. Tedesco, R.C.R. Beck, Hydrogel containing dexamethasone-loaded nanocapsules for cutaneous administration: preparation, characterization and in vitro drug release study, Drug Dev. Ind. Pharm. 36 (2010) 962–971.
[7] S.K. Sahoo, S. Parveen, J.J. Panda, The present and future of nanotechnology in human health care, Nanomedicine: N.B.M. 3 (2007) 20–31.
[8] M.L. Gordon, The role of clobetasol propionate emollient 0.05% in the treatment of patients with dry, scaly, corticosteroid-responsive dermatoses, Clin. Ther. 20 (1998) 26–39.
[9] S. Wiedersberg, C.S. Leopold, R.H. Guy, Bioavailability and bioequivalence of topical glucocorticoids, Eur. J. Pharm. Biopharm. 68 (2008) 453–466.
[10] C. Vauthier, K. Bouchemal, Preparation and manufacture of polymeric nanoparticles, Pharm. Res. 25 (2009) 1025–1058.
[11] S.R. Schaffazick, L.L. Freitas, A.R. Pohlmann, S.S. Guterres, Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos, Quim. Nova 25 (2003) 726–737.
[12] D. Milão, M.T. Knorst, W. Richter, S.S. Guterres, Hydrophilic gel containing nanocapsules of diclofenac: development, stability study and physic-chemical characterization, Pharmazie 58 (2003) 325–329.
[13] M.M. Jiménez, J. Pelletier, M.F. Bobin, M.C. Martini, Influence of encapsulation on the in vitro percutaneous absorption of octyl methoxycinnamate, Int. J. Pharm. 272 (2004) 45–55.
[14] M.P. Alves, A.R. Pohlmann, S.S. Guterres, Semisolid topical formulations containing nimesulide-loaded nanocapsules, nanospheres or nanoemulsion: development and rheological characterization, Pharmazie 60 (2005) 900–904.
[15] M.P. Alves, A.L. Scarrone, M. Santos, A.R. Pohlmann, S.S. Guterres, Human skin penetration and distribution of nimesulide from hydrophilic gels containing nanocarriers, Int. J. Pharm. 341 (2007) 215–220.
[16] K. Paese, A. Jäger, F.S. Poletto, E.P. Fonseca, B. Rossi-Bergmann, A.R. Pohlmann, S.S. Guterres, Semisolid formulation containing a nanoencapsulated sunscreen: effectiveness, in vitro photostability and immune response, J. Biomed. Nanotechnol. 5 (2009) 240–246.
[17] S.S. Guterres, M.P. Alves, A.R. Pohlmann, Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications, Drug Target Insights 2 (2007) 147–157.
[18] J. Ladermann, H. Richter, A. Teichmann, N. Otberg, U. Blume-Peytavi, J. Luengo, B. Wei, U.F. Schaefer, C.M. Lehr, R. Wepf, W. Sterry, Nanoparticles – an efficient carrier for drug delivery into the hair follicles, Eur. J. Pharm. Biopharm. 66 (2007) 159–164.
[19] G. Rao, R.S.R. Murthy, Evaluation of lipossomal clobetasol propionate topical formulation for intra-dermal delivery, Indian J. Pharm. Sci. 62 (2000) 459–462.
[20] T. Senyigit, F. Sonvico, S. Barbieri, Ö. Özer, P. Santi, P. Colombo, Lecithin/chitosan nanoparticles of clobetasol-17-propionate capable of accumulation in pig skin, J. Control. Release 142 (2010) 368–373.
[21] M. Bonneville, C. Chavagnac, M. Vocanson, A. Rozieres, J. Bebetiere, I. Pernet, A. Denis, J.F. Nicolas, A. Hennino, Skin contact irritation conditions the development and severity of allergic contact dermatitis, J. Invest. Dermatol. 127 (2007) 1430–1435.
[22] F. Di Virgilio, P. Chiozzi, D. Ferrari, S. Falzoni, M.J. Sanz, A. Morelli, M. Torboli, G. Bolignesi, R. Baricordi, Characterization of NTPDase (NTPDase 1; ecto-apyrase; ecto-diphosphohydrolase; CD39; EC 3.6.1.5) activity in human lymphocytes, Blood 97 (2001) 587–600.
[23] G. Burnstock, G.E. Knight, Cellular distribution and functions of P2 receptor subtypes in different systems, Int. Rev. Cytol. 240 (2004) 301–304.
[24] V. Ralevic, G. Burnstock, Involvement of purinergic signaling in cardiovascular diseases, Drug News Perspect. 16 (2003) 133–140.
[25] E. Kaczmarek, K. Koziak, J. Sévigny, J.B. Siegel, J. Anrather, A.R. Beaudoin, F.H. Bach, S.C. Robson, Identification and characterization of CD39 vascular ATP diphosphohydrolase, J. Biol. Chem. 271 (1996) 33116–33122.
[26] G.S. Kansas, G.S. Wood, G.S. Tedder, Expression, distribution and biochemistry of human CD39: role in activation-associated homotypic adhesion of lymphocytes, J. Immunol. 146 (1991) 2235–2244.
[27] K.E. Olson, D. Pulte, M.J. Broekman, A.E. Olson, J. Drosopoulos, N. Islam, E. Musi, A.J. Marcus, CD39/NTPDase1 variants identified in human neutrophils regulate antithrombotic activity, Blood 106 (2005) 11–16.
[28] N. Mizumoto, T. Kumamoto, S.C. Robson, J. Sévigny, H. Matsue, K. Enjyoji, A. Takashima, CD39 is dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness, Nat. Med. 8 (2002) 358–865.
[29] E.E. Clifford, K.A. Martin, P. Dalal, R. Thomas, G.R. Dubyak, Stage-specific expression of P2Y receptors, ecto-apyrase, and ecto-5′-nucleotidase in myeloid leukocytes, Am. J. Physiol. 273 (1997) 973–987.
[30] R.N. Damle, F. Ghiotto, A. Valetto, E. Albesiano, F. Fais, X.J. Yan, C.P. Sison, S.L. Allen, J. Kolitz, P. Schulman, V.P. Vinciguerra, P. Budde, J. Frey, K.R. Rai, M. Ferrarini, N. Chiorazzi, B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes, Blood 99 (2002) 4087–4093.
[31] F. Davi, H.J. Delecluse, P. Guiet, J. Gabarre, A. Fayon, O. Gentilhomme, P. Felman, C. Bayle, F. Berger, J. Audouin, P.A. Bryon, J. Diebold, M. Raphaël, Burkitt-like lymphomas in AIDS patients: characterization within a series of 103 human immunodeficiency virus associated non-Hodgkin's lymphomas. Burkitt's lymphoma study group, J. Clin. Oncol. 16 (1998) 3788–3795.
[32] H.P. Langston, Y. Ke, A.T. Gewirtz, K.E. Dombrowski, J.A. Kapp, Secretion of IL-2 and IFN-gama, but not IL-4, by antigen-specific T cells requires extracellular, J. Immunol. 170 (2003) 2962–2970.
[33] C. Goepfert, M. Imai, S. Brouard, E. Csizmadia, E. Kaczmarek, S.C. Robson, CD39 modulates endothelial cell activation and apoptosis, Mol. Med. 6 (2000) 591–603.
[34] M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, Nanoencapsulation as a way to control the release and to increase the photostability of clobetasol propionate: influence of the nanostructured system, J. Biomed. Nanotechnol. 5 (2009) 254–263.
[35] M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, Nanocapsules prepared from amorphous polyesters: effect on the physicochemical characteristics, drug release and photostability, J. Nanosci. Nanotechnol. 10 (2010) 3091–3099.
[36] E. Jäger, C.G. Venturini, F.S. Poletto, L.M. Colomé, J.P.U. Pohlmann, A. Bernardi, A.M.O. Battastini, S.S. Guterres, A.R. Pohlmann, J. Biomed. Nanotechnol. 5 (2009) 130–140.
[37] E. Martini, E. CarvalhoH, Teixeira Adsorção de oligonucleotídeos em nanoemulsões obtidas por emulsificação espontânea, Quim. Nova 30 (2007) 930–934.
[38] M.C. Fontana, M.O. Bastos, R.C.R. Beck, Development and validation of a fast RP-HPLC method for the determination of clobetasol propionate in topical nanocapsule suspensions, J. Chromatogr. Sci. 48 (2010) 637–640.
[39] M.J. Fresno Contreras, A.R. Diéguez, M.M. Jiménez Soriano, Rheological characterization of hydroalcoholic gels – 15% ethanol – of Carbopol Ultrez™ 10, Il Farmaco 56 (2001) 437–441.
[40] M.K. Kim, S.Y. Choi, Evaluation of gender difference in skin type and pH, J. Dermatol. Sci. 41 (2006) 153–156.
[41] G.S. Borghetti, M.T. Knorst, Desenvolvimento e avaliação da estabilidade física de loções O/A contendo filtros solares, Rev. Bras. Cienc. Farm. 42 (2006) 531–537.
[42] F.-Q. Hu, H. Yuan, H.H. Zhang, M. Fang, Preparation of solid lipid nanoparticles with clobetasol propionate by a novel solvent diffusion method in aqueous system and physicochemical characterization, Int. J. Pharm. 239 (2002) 121–128.
[43] J. Siepmann, N.A. Peppas, Modeling of drug release from delivery systems based on hydroxypropylk methylcellulose (HPMC), Adv. Drug Deliv. Rev. 48 (2001) 139–157.
[44] D. Quintanar-Guerrero, B.N. Zorraquín-Cornejo, A. Ganem-Rondero, E. Piñón-Segundo, M.G. Nava-Arzaluz, J.M. Cornejo-Bravo, Controlled release of model substances from pH-sensitive hydrogels, J. Mex. Chem. Soc. 52 (2008) 272–278.
[45] L.M. Brum, L.S. Lopes, N.M. Martins, J.F. Rezer, D. Araújo, G.M. Barbosa, D.A. Ferrony, I. Roggia, L.S. Trevisan, M.P. Alves, D.B. Leal, Atividade da NTPDase de linfócitos na dermatite de contato antes e após tratamento com dexametasona nanoestruturada, Lat. Am. J. Pharm. 28 (2009) 876–884.
[46] A. Böyum, Isolation of mononuclear cells and granulocytes from human blood, isolation on mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g, Scand, J. Clin. Lab. Invest. Suppl. 97 (1968) 77–89.
[47] D.B.R. Leal, C.A. Streher, T.N. Neu, F.P. Bittencourt, C.A.M. Leal, J.E.P. Silva, V.M. Morsch, M.R.C. Schetinger, Characterization of NTPDase (NTPDase 1; ecto-apyrase; ecto-diphosphohydrolase; CD39; EC 3.6.1.5) activity in human lymphocytes, Biochim. Biophys. Acta 1721 (2005) 9–15.
[48] M.M.A. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[49] R.P. Chhabra, J.F. Richardson, Non-newtonian Flow and Applied Rheology: Engineering Applications, second ed., 2008, pp. 5–12.
[50] M.A.R. Martinez, J.L.-V. Gallardo, M.M. Benavides, J.D.G. Lópes-Duran, V.G. Lara, Rheological behavior of gel and meloxicam release, Int. J. Pharm. 333 (2007) 17–23.
[51] R.C. Rowe, P.J. Sheskey, P.J. Weller, Pharmaceutical Excepients, Pharmaceutical Press, London, UK, 2001.
[52] A. Garg, D. Aggarwal, S. Garg, A. Singla, Spreading of semisolid formulations, Pharm. Technol. 26 (2002) 84–105.
[53] G.L. Flynn, P.S. Shah, S.N. Tenjarla, M. Corbo, D. DeMagistris, T.G. Feldman, T.J. Franz, D.R. Miran, D.M. Pearce, J.A. Sequeira, J. Swarbrick, J.C.T. Wang, A. Yacobi, J.L. Zatz, Assessment of value and applications of in vitro testing of topical dermatological drug products, Pharm. Res. 16 (1999) 1325–1330.
[54] G. Soslau, D. Youngprapakorn, A possible dual physiological role of extracellular ATP in the modulation of platelet aggregation, Biochim. Biophys. Acta 1355 (1997) 131–140.