Clobetasol-loaded Dermal Nanostructured Lipid Carriers for the Treatment of Imiquimod Induced Psoriasis in Mice

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Abstract

Aim: The aim of current investigation to prepare nanostructured lipid carrier system for topical delivery of clobetasol-loaded nanostructured lipid carriers (NLCs) gel for ease of application on psoriatic dermal and to evaluate its antipsoriatic efficacy compare with conventional clobetasol ointment formulation. Objective: The objective was to develop a nanogel composed of clobetasol (clobetasol -17-propionate [CP])-loaded NLCs (CP-NLCs) and to evaluate its efficiency in imiquimod (IMQ)-induced psoriasis in mice. Materials and Methods: CP-NLCs nanogel was prepared by emulsification and sonication method and optimized by design of experiments. Particle size (PS), polydispersity index (PDI), and entrapment efficiency (EE) were selected as the critical quality attributes. Antipsoriatic efficiency of CP-NLCs nanogel was evaluated by psoriatic area and severity index (PASI) score and histopathological examination in the IMQ-induced psoriasis model. Results and Discussion: Optimized formulation CP-NLCs showed that PS, PDI, and %EE were found to be 95.3 ± 2.56 nm, 0.192 ± 0.011, and 81.3 ± 2.59%, respectively. At the end of 24 h, CP-NLCs gel exhibited slow and prolonged release of CP (74.6 ± 3.82% vs. 35.1 ± 3.58) compared to CP-ointment. Furthermore, it significantly reduced the PASI score with recovery of normalcy of the mice’s skin, while the CP-loaded gel shown signs of hyper and parakeratosis at the end of the study. Conclusion: The formulated CP-NLCs gel can be a promising alternative treatment for psoriasis.

Key words: Clobetasol, imiquimod, nanostructured lipid carriers, psoriasis

INTRODUCTION

Psoriasis, a noncommunicable and autoimmune inflammatory dermal disorder, was characterized by red, scaly present on the elbows, knees, scalp, and lumbar area. Characterized symptoms frequently stated by patients include scaling, itching, erythema, burning, and bleeding. Comorbid diseases of psoriasis (e.g., Crohn’s disease, metabolic syndrome, cancer, depression, and cardiovascular diseases) further increase the physical and psychological burden. Strong evidence exists that the cell-mediated adaptive immune system, T helper 17 (T17), plays critical roles in psoriasis, while myeloid cell-produced interleukin-23 (IL-23) functions as a key cytokine for the expansion and maintenance of T17 cells. T17 cells and their downstream effector molecules, which include IL-17A, IL-22, and tumor necrosis factor-α (TNF-α)
factor hampering patient’s adherence to long-term psoriasis treatment,\(^9\) suggesting a need for development of a drug that would provide improved effectiveness but with fewer side effects. Traditional medicine, which provides front-line pharmacotherapy for billions of people worldwide, represents a possible source of a solution, although the western medical establishment is often sceptical about its efficacy due in part to a general lack of preclinical and clinical evidence.\(^9\)

For the experimental design, we used imiquimod (IMQ) for induction of psoriasis-like dermatitis. IMQ activates the toll-like receptor-7/8, which is used to treat genital warts in patients.\(^10\) IMQ-induced psoriasis-like dermatitis in BALB/c mice is mediated through the IL-23/IL-17 axis. This mouse model has been described as closely resembling human plaque-type psoriasis.\(^11\) The present study used IMQ-induced psoriasis-like dermatitis models to examine the potential therapeutic antiproliferant activity of the WCR to develop preclinical evidence of its efficacy in psoriasis therapy.

The aim of this study was to develop a nanogel composed of clobetasol propionate (CP)-loaded nanostructured lipid carriers (NLCs) gel and evaluate its potential in the psoriatic animal model. Design of experiments was used in the optimization of CP-loaded NLCs. The nanogel was prepared by dispersing the CP-loaded NLCs in Carbopol 934 gel base, and its efficacy was evaluated in IMQ-induced psoriasis in Balb/c mice based on psoriatic area and severity index (PASI) score and histopathology.

## MATERIALS AND METHODS

### Materials

Clobetasol-17-propionate was purchased from yarrow chemicals (Mumbai, India). Compritol 888 ATO was a kind gift from Gattefossé (France). Tween 80 as a surfactant, paraffin wax, and Carbopol 934 were purchased from Loba Chemie, Mumbai, India. IMQ cream (5% w/w IMQ cream) was obtained from Glenmark Pharmaceuticals (Mumbai, India). Betnovate\(^\text{®}\) (betamethasone valerate ointment, 0.1% w/w) (BMV) was procured from GlaxoSmithkline Pharmacetically Limited (Mumbai, India). Formaldehyde was obtained from HiMedia Laboratories (Thane, India). Quantikine mouse IL-17, IL-22, IL-23, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R and D Systems, Inc. (Minneapolis, USA). Methanol and acetone were purchased from Sigma-Aldrich (India). Distilled water was obtained from the in-house distillation system.

### Methods

#### Screenings of components

The solubility of clobetasol in various solid lipids (Compritol, cetyl palmitate, Sorbitan monostearate, glyceryl monostearate, cetyl alcohol, and stearic acid) were determined by adding clobetasol in increments of 1 mg until it failed to dissolve in the molten solid.\(^12\) The amount of solid lipids required to solubilize clobetasol was determined. The solubility of clobetasol in various liquid lipids and surfactants were determined by adding excess amount of drug in 5 ml of each of the lipids in glass vials. The vials were vortexed and kept at 37 ± 1.0°C in an isothermal shaker (Royal Scientific, Mumbai, India) for 48 h to reach equilibrium. The samples were filtered through 0.45 μm membrane through vacuum filtration and analyzed spectrophotometrically (Agilent 8453 ultraviolet [UV]-visible spectroscopy system) at 239 nm after appropriate dilution with methanol.\(^12\)

### Selection of a binary phase

The solid and liquid lipids were mixed in ratios of 95:5, 90:10, 85:15, 80:20, 70:30, and 60:40% w/w to establish the miscibility of two lipids using magnetic stirrer 200 rpm. The miscibility between the two components was investigated by smearing cooled sample of a solid lipid mixture onto a Whatman filter paper, followed by visual observation to determine the presence of any liquid oil droplets on the filter paper.

### Preparation of NLCs

CP-NLCs system was prepared using melt emulsification and ultra-sonication method. Initially mixing of solid and liquid lipid organic phase was prepared on a magnetic stirrer at a temperature 5°C above the melting point of solid lipid. CP-loaded NLCs preparation was prepared by adding ethanolic solution of CP (50 mg) to the above organic phase and heated until organic solvent was entirely removed from the lipid. 30 ml of preheated aqueous phase comprising 1.5% w/v surfactant was added dropwise to the lipid mixture under stirring followed by its mixing at 800 rpm for 15 min. The suspension was later subjected to probe sonication (3 mm diameter, Sonic Vibra Cell, VCX 130, USA) cycle for 10 min (30 s run and 5 s break) at 40% amplitude maintained at a temperature of 60–80°C employing oil bath. The solution so obtained was cooled in an ice bath for 15 min and later maintained under refrigerated conditions for 24 h. Thereafter, samples were lyophilized employing 2% w/v mannose (cryoprotectant) and were stored in an airtight container until further use.\(^13\)

### Characterization of NLCs

#### Mean particle size (PS), polydispersity index (PDI), and zeta potential (ZP)

The average PS, PDI, and ZP of nanocarriers were measured using PS analyzer (Malvern Zetasizer ZS 90, UK). In this, the NLCs loaded with clobetasol were dispersed in adequate quantity of distilled water to overcome the opalescence. Measurement was done at scattering angle of 90°.\(^14\)
**Percentage entrapment efficiency (%EE)**

The %EE of CP-loaded NLCs colloidal dispersion was analyzed indirectly by determining the amount of unentrapped clobetasol existed in the aqueous phase of colloidal dispersions; the calculation was done using the following equations:

\[
\%EE = \left( \frac{\text{Total amount of CP} - \text{Amount of free CP}}{\text{Total amount of CP}} \right) \times 100
\]

The unentrapped clobetasol was separated by centrifugation and filtration. The aqueous colloidal dispersion of CP loaded NLCs was centrifuged at 11,000 rpm for 15 min maintained at 4°C. After centrifugation, the supernatant dispersion was measured using UV-visible spectrophotometer at 239 nm.\[15\]

**Formulation of CP-NLCs gel**

CP-NLCs colloidal dispersion was converted into the gel using Carbopol (CP 934) as a gelling agent. 1% w/w Carbopol 934 was dissolved in distilled water and stirred for 15 min at 1000 rpm. Consequently, calculated amount of freshly prepared CP-NLCs colloidal dispersion was mixed for 10 min and then neutralized by drops of triethanolamine until pH 5.5. The formulated gel was further allowed to stand overnight to remove entrapped air.\[16\]

**Preparation of ointment**

CP-ointment was formulated by simple melt dispersion. In which, paraffin wax and stearyl alcohol were melted under heating to get a uniform melt into this BHT was added and mixed for some time. Finally, the CP is added and blends thoroughly to get uniform spreading and evaluate drug release.\[17\]

**In vitro drug release studies**

*In vitro* release studies of CP loaded NLCs gel, ointment and solution were carried out using modified Franz diffusion cell with a receptor volume capacity of 12.5 ml using cellulose acetate membrane (MWCO – 12,000–14,000 Da, pore size – 2.4 nm, HiMedia, Mumbai, India) and phosphate-buffered saline of pH 7.4 as a dialyzing medium. The membrane was soaked in double-distilled water for about 12 h, before mounting the membrane (diffusion area 1.95 cm²) in the Franz diffusion cell. The pretreated membrane was placed on the modified Franz diffusion cell filled with phosphate buffer (pH 7.4) in the receptor compartment. The whole assembly was placed on the magnetic stirrer at 300 rpm, and temperature was maintained at 32.0 ± 0.5°C. The samples (CP loaded NLCs gel, ointment, and solution) equivalent to 200 µl gel were kept over the membrane in donor compartment and stirred. 2 ml samples were withdrawn from the receptor compartment at predetermined time intervals (0, 6, 8, 10, 12, and 24 h) and the same volume was refilled with diffusion medium. The samples were filtered and analyzed using UV spectrophotometer at 239 nm after appropriate dilutions. Percent drug release was calculated and graph was plotted between percent drug releases against time. Release studies were performed in triplicate for each formulation.\[18\]

**IMQ-induced psoriasis in mice**

8 to 11-week-old BALB/c male mice (weighing approximately 15–20 g each) were purchased from the Sri Venkateshwara Animal House, Bengaluru. The animals were housed under strict hygienic conventional conditions in cages at 22 ± 1°C with 50–60% relative humidity and were subjected to a 12 h light/12 h dark cycle in the laboratory animal house. The mice were acclimatized for 1 week and were provided with commercial rodent diet and water *ad libitum* before being used in the research. Before and during the experimental period, all mice were assessed for their health status, including food and water intake, body weight, behavioral signs, respiratory patterns, and cardiovascular signs. Experiments were conducted according to international and national guidelines for ethical conduct in the care and use of animals and were approved by the Animal Ethics Committee of the Krishna Teja Pharmacy College, Tirupati (Protocol approval number: IAEC no. 16/KTPC/IAEC/2018:1/03/2018). Histological and clinical features of dermatitis in mice induced by IMQ are similar to psoriasis manifestations, including erythema, scaling, and inflammation.\[11\] The dorsal skin of the mice was shaved, and a commercially available 5% IMQ cream was applied. The mice were randomly divided into five groups of five animals and received the treatment. Briefly, on day 1 and continuing for 6 consecutive days, mice in all groups except for the normal group received a daily topical dose of 62.50 mg of the IMQ cream on the shaved area of their backs. On the right ear, 5% IMQ cream was applied at a dose of 20 mg/cm².

- **Group I**: Normal (sham control) group received only Vaseline on the shaved dorsal skin surface and left ear pinna
- **Group II**: Negative control group received a daily topical dose of 5% (62.5 mg) IMQ cream on shaved back and right ear pinna (left ear pinna was untreated)
- **Group III**: Positive group received IMQ + betamethasone valerate at 100 mg/day
- **Group IV**: CP-ointment group received IMQ + CP-ointment 50 mg/day
- **Group V**: CP-NLCs gel group received IMQ + CP-NLCs gel 50 mg/day.

**Sample collection**

All the mice in Groups I–V were sacrificed at the end of the experiment by an intraperitoneally injected overdose (50–90 mg/kg) of pentobarbital. Pentobarbital was used in this study because its profile on the sleeping time and loss of righting reflex has been well established after pentobarbital administration. They were then sacrificed on day 6 at the
Psoriasis area and severity index of skin inflammation

PASI scores were determined by evaluating the degree of erythema, thickening, and scaling on the affected dorsal skin surface and ear pinna. PASI for each was measured on a four-point scale (0 = None; 1 = Slight; 2 = Moderate; 3 = Marked; and 4 = Very marked). The severity of skin inflammation was measured by the combined scores (erythema plus scaling plus thickening) giving a range of scores of 0–12. Ear thickness was measured twice every other day using digital calipers (BEC, China). An increase in ear thickness was used to indicate the extent of epidermal proliferation and inflammation.

Histopathological examinations

All the groups’ mice tissue samples were fixed in 10% neutral buffered formalin before processing and embedding in paraffin blocks. Sections of the samples were prepared at 4 µm thickness using a rotary microtome and were stained with hematoxylin and eosin (H and E) stain; then they were observed under a microscope using a digital camera system.

Changes of spleen in mice

The spleen from each mouse was isolated, and a photograph was taken before being weighed. Splenomegaly was evaluated by calculating the ratio of the weight of the spleen to the bodyweight.

Assay of cytokines production

To measure cytokine levels in serum, mouse blood was collected at 24 h after the final treatment using the cardiac puncture method; serum was stored at −70°C until analysis. To measure cytokine levels in skin tissue, the central dorsal skins of the mice were removed and stored at −80°C. The skins were later homogenized in tissue protein lysis buffer (Bio Basic Inc., Canada) at 4°C, and the supernatants were stored at −80°C until analysis. The concentrations of IL-17A, IL-22, and IL-23 in the mouse serum and skin tissue were measured using mouse IL-17A, IL-22, and IL-23 ELISA MAX Deluxe (BioLegend, USA). ELISA was performed in accordance with the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using the trial version of GraphPad Prism version 6.01 software (GraphPad Software, San Diego, CA). The level of statistical significance was determined by analysis of variance followed by Bonferroni’s test for multiple comparisons. The mean differences were considered significant in all experiments valued at *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Percentage Entrapment efficiency (%EE)

The interaction effect of lipid to drug ratio with surfactant concentration and sonication time showed a positive effect. Formulation with lipid:drug ratio of 7.5:1 showed greater drug percentage entrapment efficiency, i.e., 81.3±2.59%.

**In vitro release study**

The cumulative percentage of drug release of CP from CP solution, CP ointment, and CP loaded NLCs gel was investigated in vitro over a period of 24 h. Each sample was analyzed in triplicate, and release curves are shown in Figure 1. The drug release from CP solution was faster with 94.5±2.98% release of CP within 7 h, whereas drug release from the ointment and NLCs gel was comparatively slow; it showed 35.1±3.58 and 74.6±3.82%, respectively in 24 h.

**Effect of IMQ-induced psoriasis-like dermatitis in mice**

Characteristics and health status such as food and water consumption, and body weight were observed in all the groups during 6th-day study. Compared to their respective initial weights slight decline was observed in all groups until 4th day of study. From 4th day to 6th day, slight increase in body weight was observed in case of control and treatment groups [Figure 2a]. Behavior signs, respiratory patterns, and cardiovascular signs of all mice were normal throughout the experimental period. Two days after starting IMQ application, it was observed that both the dorsal skin and the right ear pinna of the mice exhibited signs of erythema, scaling, and thickening. Thereafter, the intensity of psoriasis-like symptoms of Group II mice progressively increased in severity until the end of the treatment (day 6). However, mice in Group I treated daily with Vaseline did not show any signs of inflammation on dorsal skin or right ear pinna, Group II treated with negative control, Group III treated with positive control, Group IV treated with CP-ointment, and Group V CP-loaded NLCs gel and their PASI scores (erythema, scaling, and skin thickening), depicted in Figure 2b,c and d. The PASI scores reached peak intensity at the 6th day after IMQ treatment which indicates successful induction of psoriasis-like dermatitis in the IMQ-treated mice. Ear thickness of the mice was measured as an independent parameter of skin inflammation [Figure 2e]. The size and weight of the spleen were markedly enlarged in IMQ-induced psoriasis mice. The ratio of spleen weight to body weight was significantly decreased in the CP-NLCs gel [Figure 2f].

**Phenotypical observations**

Analysis of phenotypical presentations of CP-NLCs gel IMQ-induced inflammation accompanied by structural feature characteristic of psoriasis found that dorsal skin started to display erythema, thickening, and scaling beginning 2 days after the first IMQ application, with maximum inflammatory severity occurring at 5th day. The

![Figure 1: In vitro drug release profile from clobetasol propionate-nanostructured lipid carriers gel, ointment, and solution in phosphate buffer (pH 7.4). Values were expressed as mean ± standard deviation (n = 3)](image)
intensity of the psoriasis-like symptoms in the IMQ-only treated group (Group II) was observed to have steadily increased from day 1 to day 6. However, there was a statistically significant decrease in psoriasis-like symptoms beginning at day 5th, the 2nd day after initiation of treatment with betamethasone valerate (Group III), CP-ointment (Group IV), and CP-NLCs gel (Group V). These symptoms consistently declined until 6th day, the end of the treatment. Comparing with the IMQ-treated group, all treated groups shown a significant inhibitory effect on IMQ-induced psoriasis-like dermatitis.

**Histopathological analysis**

Analysis of H and E stained sections from the IMQ-treated dorsal skin was found to be in line with the phenotypical observations and PASI score results. The dorsal skin and right ear pinna sections of the IMQ-treated mice showed significantly increased acanthosis, hyperkeratosis of the epidermis, and inflammatory infiltration. However, the dorsal skin and right ear pinna of control mice sections were normal in both epidermis and dermis [Figure 3]. Interestingly, epidermal thickness was greatly reduced in the positive
Psoriasis is a Th1/Th17 mediated inflammatory process associated with overexpression of Th1 and Th17-associated cytokines (IL-17, IL-22, IL-23, and TNF-α) leading to inflammation and keratinocyte hyperproliferation. IMQ-induced psoriasis model mimics biochemical parameters characteristic of human psoriatic lesions. Topical application of the IMQ cream was reported to increase the levels of cytokines such as IL-17, IL-22, IL-23, and TNF-α in the treated skin tissues (Van der Fits et al., 2009).

**ELISA**

control and CP-NLCs gel-treated mice compared with the IMQ-treated mice.

**Figure 3:** Phenotypical and histopathological features of the skin during efficacy studies on imiquimod-induced psoriatic plaque model. (a and a’) Normal group, (b and b’) negative control group, (c and c’) positive control group, (d and d’) clobetasol propionate (CP)-ointment group, and (e and e’) CP-nanostructured lipid carriers gel group. Each image is representative of respective group (n = 5)

**Figure 4:** Determination of pro-inflammatory interleukin levels in skin homogenates using enzyme-linked immunosorbent assay. Results are presented as group means ± standard deviation (n = 5) for (a) interleukin (IL)-17, (b) IL-22, (c) IL-23, and (d) tumor necrosis factor α levels. ***P < 0.001 (vs. Sham), *P < 0.05 (vs. Sham), ###P < 0.001 (vs. Negative control), #P < 0.05 (vs. Negative control)
The present study was evaluated to determine the potency of dermally applied CP-NLCs gel was estimated by determining the levels of IL-17, IL-22, IL-23, and TNF-α cytokines present in the skin homogenate collected on the 7th day. The T17 cytokines play an important role in psoriasis and the compared to sham group, and negative control exhibited significant elevation of cytokine levels of IL-17, IL-22, IL-23, and TNF-α. In this present study, CP-loaded NLCs gel showed 2.9-folds, 3.8-folds, and 2.1-folds reductions in IL-17, IL-22, and IL-23 levels, respectively, whereas clobetasol ointment showed 1.5-folds, 2.4-folds, and 1.3-folds reductions in IL-17, IL-22, and IL-23 levels, positive control group which was treated with betamethasone showed 2.2-folds, 2.7-folds, and 1.6-folds reduction in IL-17, IL-22, and IL-23 levels, respectively, compared to negative group. TNF-α levels were reduced in CP-loaded NLCs group, ointment and positive control group by 2.2-folds, 1.3-folds, and 1.5-folds, respectively, compared with negative control [Figure 4]. Treatment with the CP-NLCs gel decreased the cytokines levels which confirmed the efficacy of formulation in treating psoriasis.

CONCLUSION

In the present study, the CP-loaded NLCs based nanogel was successfully prepared by using Compritol and oleic acid as solid and liquid lipid using emulsification and sonication technique. The dermal delivery of CP to the psoriatic skin by means of NLCs could possibly minimize the side effects. This preclinical evidence suggests that CP-loaded NLCs gel may have the pharmacological actions of regulating T17 related cytokines, making it potentially useful as an alternative therapeutic strategy for psoriasis. Further investigation is needed to evaluate acute and chronic toxicities in animal models before performing a clinical study. Furthermore, CP loaded NLCs were capable of reducing psoriatic lesions on mice skin.

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AUTHORS’ CONTRIBUTIONS

All the authors have contributed equally.

ETHICAL APPROVAL

All experimental procedures were performed in accordance with the ethical guidelines for the study and were approved by the Institutional Animal Ethical Committee (IAEC no. 16/KTPC/IAEC/2018/1/03/2018), Krishna Teja Pharmacy College, Chadalawada Nagar, Tirupati, Andhra Pradesh, India.

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