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CLOBETASOL-LOADED SOLID LIPID NANOPARTICLES TOPICAL GEL FOR PSORIASIS: IMIQUIMOD INDUCED MICE PSORIATIC PLAQUE MODEL

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ABSTRACT:
The aim of the current investigation was to prepare and investigate the potential of Clobetasol (CP) loaded solid lipid nanoparticles based gel (SLN-gel) for the dermal delivery and to evaluate its anti-psoriatic efficacy by comparing with conventional ointment formulation. This study will provide an insight about the use of nanocarriers. SLNs loaded with CP for the effective treatment of psoriasis. Clobetasol-loaded SLNs were formulated by emulsification-homogenisation method and were characterized for particle size, polydispersity and percentage entrapment efficiency using DLS technique. The optimized SLNs were loaded into gel and evaluated for drug release, spreadability. Anti-psoriatic efficacy of the SLNs gel was evaluated in imiquimod (IMQ) induced psoriatic plaque model by comparing with prepared conventional ointment formulation (0.05% w/w CP). Optimized SLNs attains a particle size of 133.3±3.66 nm, polydispersity index (PDI) 0.179±0.081, percentage entrapment efficiency of 78.1±1.11% and Zeta potential of -36.2±0.11mV. The prepared SLNs gel was then compared with conventional ointment for drug release, and efficacy. Topical application of CP-loaded SLNs gel on IMQ induced psoriatic plaque model reduced the symptoms of psoriasis which was assessed by both Psoriasis area severity index (PASI) scoring and enzyme-linked immunosorbent assay.To conclude CP-loaded SLNs gel enhances dermal delivery and was efficacious in management of psoriasis when compared to conventional ointment.

Keywords: Solid lipid nanoparticles, Clobetasol, Imiquimod, Anti-psoriatic, ELISA.

1. INTRODUCTION

Psoriasis is a chronic autoimmune skin disorder that was characterized by erythematous lesions with epidermal hyperplasia (acanthosis) and extensive inflammation (Hamminga et al., 2006). Further, the corneocytes (dead keratinocytes) of the stratum corneum tend to retain their nuclei (parakeratosis) due to uncharacteristic terminal differentiation of the keratinocytes which manifests as red, scaly and raised patches of lesions on skin. There is also dermal infiltration of inflammatory cells such as dendritic and T cells, which results in the activation of cytokines including IL23, IL17, IL22, TNFα and NFκB that promote the chronic inflammation (Works et al., 2014).

There is burgeoning research evidence that strongly suggests that the IL1 family of cytokines crucial roles in the pathogenesis of psoriasis. Particularly, the IL36 cytokine family (IL36 α (IL1F6), IL36 β (IL1F8) and IL36 γ (IL1F9) has been reported to augment the expressions of TH17 cytokines such as IL17A in a feedback mechanism that results in the activation of IL6, IL23 and TNFα (Carrier et al., 2011; Towne et al., 2012). Noteworthily, diverse studies have shown the induction of IL36 cytokines in both human psoriatic skin and psoriatic-like mouse models. In this study the novel IL36 α and its receptor, IL36R, have been demonstrated extensively to induce the expression of MAP-kinase and NFκB which causes chronic inflammation associated with psoriasis (Derer et al., 2014).

Glucocorticoids (GCs) were highly effective drugs which were widely used in dermatology for the treatment of inflammatory diseases. However, severe adverse effects often accompany which their long-term use (Zöller et al., 2008; Schoepe et al., 2006). Clobetasol- 17-propionate (CP) was considered as most potent drug of choice among the currently available topical corticosteroids. It is currently approved for topical administration in different dosage forms such as cream, gel, solution, ointment, and foam (Franz et al., 2003).

Solid lipid nanoparticles (SLNs) were novel colloidal carrier systems composed of high melting point solid lipid core, coated by surfactants (Ghadiri et al., 2012; Singh et al., 2014). SLNs offer distinct advantages like negligible skin irritation, controlled release and protection of substances as it uses physiologically tolerated, non-irritative and non-toxic lipids (Prow et al., 2011; Singh et al., 2013). SLNs were found to be safe for administration to inflamed and damaged skin. Moreover, small size of the lipid particles ensures its close association with stratum corneum increasing the quantity of the drug penetrating into the skin. Solid lipid matrix of SLN also permits sustained release of drugs from the nanocarrier (Schafer-Korting et al., 2007; Singh et al., 2014**).** SLNs reduce trans epidermal water loss decreases trans epidermal water loss and favors the drug penetration through the stratum corneum and enhances dermal localization (Prow et al., 2011). The transdermal drug delivery is a viable approach for consideration because of its non-invasive feature and it circumvents the hepatic-portal circulation, which makes it an ideal delivery method for drugs that are highly susceptible to first pass metabolism. However, the stratum corneum is composed of lipids (ceramides, fatty acids and cholesterol) that form highly impermeable bilayered matrix which makes it impossible to permeate by macromolecules and hydrophilic drugs.

The aim of this study was to develop a nanogel composed of CP-loaded SLNs and evaluate its potential in the psoriatic animal model. Design of Experiments (DoE) was used in the optimization of CP-loaded SLNs. The nanogel was prepared by dispersing the CP-loaded SLNs in Carbopol 934 gel base and its efficacy was evaluated in imiquimod-induced psoriasis in Balb/c mice based on Psoriatic Area and Severity Index (PASI) score and histopathology.

2. 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. Imiquimod cream (5% w/w IMQ cream) was obtained from Glenmark Pharmaceuticals (Mumbai, India). Betnovate® (betamethasone valerate ointment, 0.1% w/w) (BMV) was procured from GlaxoSmith Kline Pharmaceutical Limited (Mumbai, India). Formaldehyde was obtained from HiMedia laboratories (Thane, India). Quantikine mouse interleukin-17 (IL-17), interleukin-22 (IL-22), interleukin-23 (IL-23) and tumor necrosis factor- α (TNF- α) ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, USA). Methanol, and Acetone were purchased from Sigma–Aldrich (India). Distilled water was obtained from the in-house distillation system.

Method

Preparation of CP-loaded SLNs

SLNs were formulated by emulsification-homogenisation method. Formulation procedure was divided into two portions one portion contains drug and lipid, while the second portion contains an aqueous solution of surfactant and stabilizer. Lipid mixture was melted 5ºC above the melting point of lipid and drug was dispersed into it. Aqueous phase was heated to same temperature as like lipid phase. When both portions attain equilibrium, the aqueous phase was incorporated into lipid phase and emulsified using high speed homogenizer (IKA® T-10 basic Ultra-Turrax®, Germany) at 10,000 rpm for 15min. The obtained pre-emulsion was ultrasonicated using a probe Sonicator (VibracellTM 700W; Sonics, USA) at 80% amplitude for duration of about 5min. Cycles were repeated alternatively until it attains uniform particle size. The resulting nanoemulsion was cooled down in an ice bath to produce nanoparticle dispersion. Different formulations were prepared by varying the critical process variables (Das et al., 2011).

Characterization of CP SLNs

Particle size, Polydispersity index and Zeta potential measurement

Particle size, polydispersity index (PDI) and zeta potential of the SLNs were measured by dynamic light scattering technique using Malvern Zetasizer Nano ZS90 (Malvern Instruments, UK). CP SLNs colloidal dispersions were diluted 10 times with double distilled water. Particle size and PDI measurements were performed by taking 1 ml of diluted formulation into polystyrene cuvettes and disposable folded capillary cell for zeta potential at 25ºC, to ensure that the light scattering intensity was within the instruments sensitivity range. Each sample was analyzed in triplicate and the results were shown as mean ± standard deviation.

% Entrapment efficiency

The entrapment efficiency (%EE) of formulated CP SLN was determined by measuring the concentration of unentrapped drug in the lipidic dispersion by using centrifugation method. The SLNs Samples were subjected to centrifugation at 10000 rpm for 15 min (Remi Centrifuge Pvt. Ltd., India) and the amount of Clobetasol propionate Supernatant was collected, suitably diluted with methanol and analyzed for free drug content by UV spectroscopy at 239nm. %EE was calculated by following equation.

$$\%EE = \frac{Total\ amount\ of\ CP - Amount\ of\ free\ CP}{Total\ amount\ of\ CP}$$

Transmission electron microscopy

The morphological characters of CP loaded SLNs were observed with transmission electron microscopy (TEM; Philips, Tecnai 20, Holland). A drop of the diluted sample was placed on the surface of carbon coated copper grid and stained with a drop of 1% (w/w) aqueous solution of phosphotungstic acid (negative stain) for 30s. Excessive staining solution was washed out by filter paper and a thin aqueous film was left on the surface. After staining, samples were dried at room temperature for 10 min to perform out investigation (Rawat et al., 2007).

Formulation of CP-SLN topical gel

Direct application of SLNs is not suitable as they cannot reside on skin for longer duration which is crucial for skin disorders like psoriasis. In such disorders, the topical ointment/gel based delivery is essential. CP-SLNs dispersion was converted into gel carrier system using Carbopol (CP 934) as gelling agent. 1% w/w Carbopol 934 was dissolved in distilled water, stirred for 15 min at 1000 rpm. Subsequently, calculated amount of freshly prepared CP-SLNs dispersion was added and mixed for 10 min, then neutralized by drops of triethanolamine until pH 5.5. Prepared gels were further allowed to stand overnight to remove entrapped air (Kesharwani et al., 2016).

Characterization of Clobetasol-loaded SLNs gel Spreadability

Spreadability is an essential property of any topical formulation which assists in uniform application of actives to the affected area. The spreadability of the Clobetasol-loaded SLNs gel was evaluated by parallel plate method as reported. The study was performed by taking 0.5 g of SLNs gel and placing it on the glass plate at center, another glass plate was concentrically placed above it. The gel was pressed with known weights starting from 5 to 200 g at interval of 30 s between each weight. Spreadability of the formulation was measured by measuring the diameter of the formulation in two perpendicular directions (Marchiori et al., 2010). The spreadability factor was calculated using Equation (2).

$$Sf = \frac{A}{W}$$

where S_f (cm2/g) is the spreadability factor, defined as the ratio of the maximum spread area (A) in cm^2 after the addition of the sequence of weights and the total weight added (W) in gm. Spreadability profile was obtained by plotting the spreading area on y-axis and weights added on x-axis.

Preparation of ointment

Clobetasol ointment was prepared by simple melt dispersion. Briefly, paraffin wax and stearyl alcohol were melted under heating to form a uniform melt. To this BHT was added and mixed for some time. Finally, the drug was added and mixed thoroughly to aid uniform drug distribution. The formulation was then stirred at room temperature until it is cooled (Aukunuru et al., 2007). The obtained formulation was characterized for spreadability and drug release.

In vitro drug release studies

In vitro release studies of CP loaded SLNs 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 PBS of pH 7.4 as a dialyzing medium. The membrane was soaked in double-distilled water for about 12 h, prior to mounting the membrane (diffusion area 1.95cm2) in the Franz diffusion cell. 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 SLNs gel, ointment and solution) equivalent to 200µl gel were kept over the membrane in donor compartment and stirred. 2ml 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 release against time. Release studies were performed in triplicate for each formulation.

Anti-psoriatic efficacy studies in BALB/c mice

In vivo Imiquimod-induced psoriatic plaque model

IMQ induced psoriatic plaque model was developed in male BALB/c mice of 8 to 11 weeks age and weight of about 20 - 25 g (Sun et al., 2013). Animals were housed under controlled environmental conditions (temperature 23 ± 1 °C, humidity 55 ± 5%, and 12 h light/dark cycles) with food and water ad libitum. The animals were divided into five groups and notified i.e., normal (Sham), negative control, positive control, Ointment, SLN gel (Table I). All the animals were depilated on their dorsal side the day before starting study. Sham group was left untreated and for remaining groups, 62.5 mg of commercially available IMQ cream (5% w/w) was applied on the depilated skin and left ear for 6 consecutive days. The animals (treatment groups) were treated with respective formulations from third day of the study and continued till sixth day. Positive control group received treatment with 100 mg/d of betamethasone valerate (BMV) ointment (0.01% w/w) while other two groups received treatment with 50 mg/d of 0.05% w/w of Clobetasol loaded SLNs gel and 50 mg/d of 0.05% w/w of Clobetasol ointment. Change in body weight of the animals was noted on 0, second, fourth, and sixth day and Psoriasis area severity index (PASI) scores were assessed for all groups of animals during the treatment. All group animals were sacrificed on the seventh day by cervical dislocation method; thickness of both ears was measured by screw gauge and spleen weights were recorded for all the animals. Skin samples were collected, washed with buffer solution and were fixed in 10% v/v formalin solution for histopathological examination. Portion of skin sample was collected and stored at 80°C for performing ELISA (to determine cytokines level).

Table I. Treatment protocol followed for efficacy studies in imiquimod induced psoriasis model.

	Days						
Group	0	1	2	3	4	5	6
Sham	--	--	--	--	--	--	--
	--	--	--	--	--	--	--
	--	--	--	--	--	--	--
Negative control	--	IMQ[a]	IMQ	IMQ	IMQ	IMQ	IMQ
	--	--	--	--	--	--	--
	--	--	--	--	--	--	--
Positive control	--	IMQ[a]	IMQ	IMQ BMV[b]	IMQ BMV	IMQ BMV	IMQ BMV
	--	--	--	--	--	--	--
	--	--	--	--	--	--	--
CP ointment	--	IMQ[a]	IMQ	IMQ CP ointment	IMQ CP ointment	IMQ CP ointment	IMQ CP ointment

	--	--	--	--	--	--	--
	--	--	--	--	--	--	--
CP-SLNs	--	IMQ[a]	IMQ	IMQ CP-SLNs	IMQ CP-SLNs	IMQ CP-SLNs	IMQ CP-SLNs
	--	--	--	--	--	--	--
	--	--	--	--	--	--	--

a Imiquimod.
b Betamethasone valerate

Table II. Optimization of formulation parameters for solid lipid nanoparticles.

Optimization Parameters				**Particulate Characters**		
S.No	**Surfactant Concentration (%w/v)**	**Drug to lipid ratio (w/w)**	**Homogenization time**	**Particle size (nm)**	**PDI**	**%EE**
1	2		5	261.5±5.01	0.310±0.077	53.1±3.26
	3	1:3	10	175.4±2.35	0.251±0.052	64.1±4.1
	4		15	251.7±4.21	0.432±0.039	39.8±3.12
2	2		5	241.1±3.26	0.295±0.056	57.5±2.36
	3	1:4	10	133.3±3.66	0.179±0.081	78.1±1.11
	4		15	262.2±3.97	0.457±0.061	46.3±3.25
3	2		5	288.7±4.13	326±0.08	63.2±3.22
	3	1:5	10	209.±5.63	0.284±0.035	78.1±3.45
	4		15	252.0±2.06	0.440±0.065	54.5±2.81

Values are expressed as mean ± SD (n = 3).

PASI scoring

The clinical scoring method for the Psoriasis Area and Severity Index (PASI) was used to score the severity of the inflammation induced on the dorsal regions of the mice daily based on the extent of scaling and erythema, epidermal thickening. The scoring was recorded on the 0, second, fourth, and sixth day of treatment. The scores were assigned from 0 to 4 scale based on the severity of erythema (redness), scaling and thickening of skin i.e 0 (none), 1 (slight), 2 (moderate), 3 (severe), and 4 (very severe). These scores were plotted time points on x axis and PASI score on y-axis.

Histology

Skin samples collected at the end of the study which were fixed in formalin were given for histopathology examination. This study was performed to determine the pathological changes like acanthosis, inflammatory infiltrates, hyperkeratosis, and parakeratosis that occurred during the treatment period. Briefly, formalin- fixed, paraffin-embedded skin sections were deparaffinized, rehydrated and stained with hematoxylin and counterstained with eosin. The stained skin sections were mounted and viewed with the Olympus BX40 light microscope equipped with a computer- controlled digital camera (DP71, Olympus Center Valley, PA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to know the cytokines like IL-17, IL-22, IL-23, and TNF-levels in the treated groups. The skin samples collected at the end of the psoriatic efficacy study were stored in -80°C until used for the ELISA. The collected tissue samples were homogenized in double amount of an extraction buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% TritonTM X-100) with help of tissue homogenizer (Remi Electrokinetic, Ltd., Mumbai, India) at 4000 rpm for 5 min. The homogenates samples were centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant were collected and stored in -80°C until analyzed. The samples were analyzed for levels of cytokines using quantitative Mouse ELISA kits (R&D Systems, Minneapolis, MN)

as per manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using 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 (ANOVA) followed by Bonferroni's test for multiple comparisons. The mean differences were considered significant in all experiments valued at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3. RESULTS AND DISCUSSION

Selection of preparation method

Emulsification-homogenization method was selected to formulate CP loaded SLNs. The particulate characters were tabulated in (Table.II). As per the results Particle size, polydispersity index and zeta potential were found to be 133.3±3.66 nm, 0.179±0.081, 78.1±1.11% and -36.2±0.11 mV respectively.

Optimization of surfactant concentration

Clobetasol-loaded SLNs were optimized for different surfactant concentrations from 2 to 4% w/v. The lowest particle size, poly disperse index and % entrapment efficiency was obtained with Tween 80 at 2%, 3%, and 4% w/v by keeping homogenization speed at 10,000 rpm for 10 min with solid lipid to drug ratio constant (1:4) as shown in (Table 2). Prepared SLNs with three concentrations of Tween 80 at 2 and 4% w/v concentration, higher particle size, PDI and lower % entrapment efficiency was observed and the lower particle size, PDI and higher % entrapment efficiency were shown Tween 20 at 3% w/v. Based on the particle size and PDI observations formulation with Tween 20 (3% w/v) was selected for further studies to keep surfactant concentration minimum.

Optimization of solid lipid and drug ratio

Clobetasol-loaded SLNs were optimized for the ratio of solid lipid to drug. Batches for optimization were prepared in three ratios 3:1, 4:1, and 5:1. The ratio having lowest particle size, PDI and higher % entrapment efficiency was selected for further studies with Tween 80 at 3% w/v concentration and 10 min homogenization as constant. The lowest particle size, PDI and higher % entrapment efficiency for the batches were with ratio 4:1 i.e. 133.3±3.66 nm, 0.179±0.081 and 78.1±1.11% respectively.

Optimization of homogenization time

Effect of homogenization time on particle size, PDI and % entrapment efficiency was studied at homogenization speed of 10,000 rpm. The homogenization time was varied from 5 to 15 min. From the study it was observed that, the particle size reduces with increase in homogenization time for 10 min. After this, there was an increase in particle size, PDI and % entrapment efficiency.

Morphology of SLNs

Size and shape of the optimized batch of nanoparticles were evaluated by TEM. TEM images of the SLNs confirmed the oval and nearly spherical shape of nanoparticles with narrow size distribution. They further confirmed non-aggregation of nanoparticles (Figure 1). The diameters of the nanoparticles observed in the micrographs were in good agreement with data obtained from Malvern particle size analyzer.

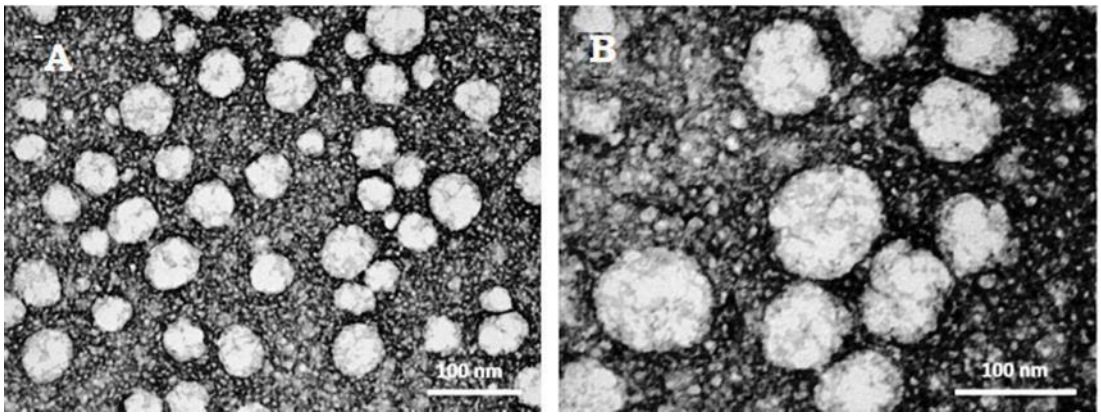


Figure 1. CP loaded SLNs (.optimized formulation) visualized by transmission electron microscopy. (a) Overall view showing particle polydispersity, (b) magnified view showing particle internal structure.

Entrapment efficiency

The interaction effect of lipid to drug ratio with surfactant concentration and homogenization time showed a positive effect. Formulation with lipid: drug ratio of 4:1 showed greater drug entrapment, i.e., 78.1%. A higher %EE could be due to the presence of higher amount of lipid which provides additional space for a drug molecule to embed in, thereby decreasing total surface area. This can lead to reduction in the diffusion rate of the solute molecule as the viscosity of the lipidic phase is higher and thus showed higher %EE s compared to others. % EE was found to increase with the increasing amount of lipid to drug molar ratio. Thus, % EE was found to be mainly dependent on the drug: lipid ratio of the formulation.

Spreadability

The CP ointment and CP-SLNs were evaluated for spreadability and comparison of spreadability of the ointment and gel with increasing weights was shown in (Figure 2)**.** Initial spreading area for CP ointment was 1.7 ± 0.32 cm^2 whereas CP-SLNs Gel showed a spreading area of 6.8 ± 0.23 cm^2. After the addition of 200 g weights to the formulations, the spreading area was 4.46 ± 0.35 cm^2 for CP ointment and it was 21.9 ± 0.15 cm^2 for CP-SLNs-Gel. Significant difference was observed between spreadability of ointment and SLNs gels. The spreading area of the CP-loaded SLNs gel was higher as compared to the ointment which means gel can be applied to skin with less force of application.

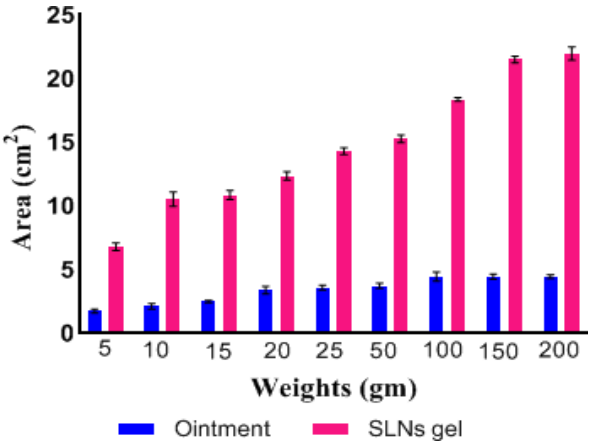


Figure 2. Comparison of spreadability profile of CP ointment and CP-SLNs gel. Values are expressed as mean ± SD (n = 3).

In-vitro release study

The cumulative percentage of drug release of CP from CP solution, CP ointment and CP loaded SLNs gel was investigated in vitro over a period of 24h. Each sample was analyzed in triplicate and release curves have been shown in (Figure 3). The drug release from CP solution was faster with 93.9 ± 3.84% release of CP within 7h. Whereas drug release from the ointment and SLNs gel was comparatively slow, it showed 33.9 ± 4.29 and 67.3 ± 3.36%, respectively in 24 h. As both the solution and ointment do not have the drug in encapsulated form, the drug got oxidized and hence there is a reduction in concentration. In SLNs gel, drug is protected from oxidation as it is encapsulated along with presence of anti-oxidants and hence such trend was not observed in release profile of SLNs gel. For topical application, the formulations were expected to retain on skin for 24 h, for this reason, the in vitro release was studied for 24 h. To understand the best fit model and possible mechanisms governing the drug release in CP-SLNs formulation, various kinetics models were fitted including zero-order (r^2 = 0.906), first-order (r^2 =0.994), Higuchi (r^2 = 0.978) and Korsmeyer–Peppas models (r^2 = 0.980). Peppas model was found to be best fit model for SLNs gels. Further analysis of the diffusion exponent (n) in Korsmeyer –Peppas equation revealed that values of n was greater than 0.5 in the formulation, a non Fickian diffusion, as a combination of both diffusion and erosion controlled rate release.

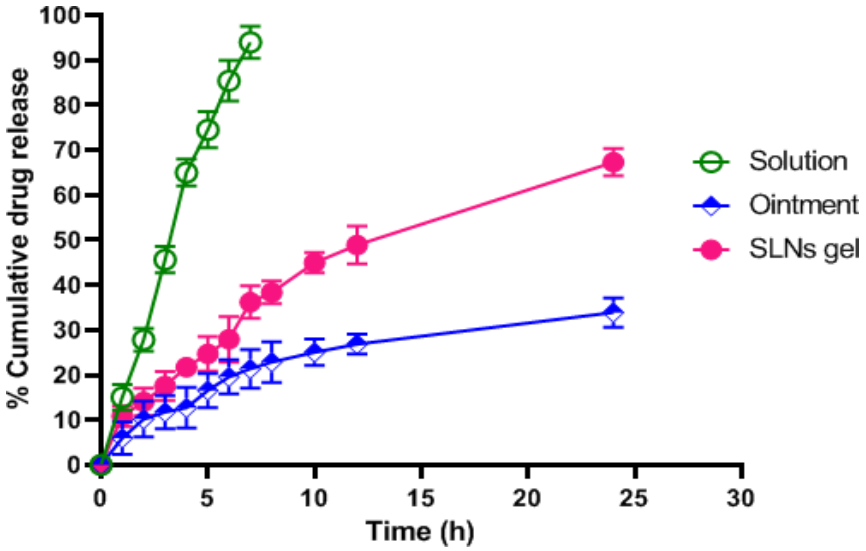


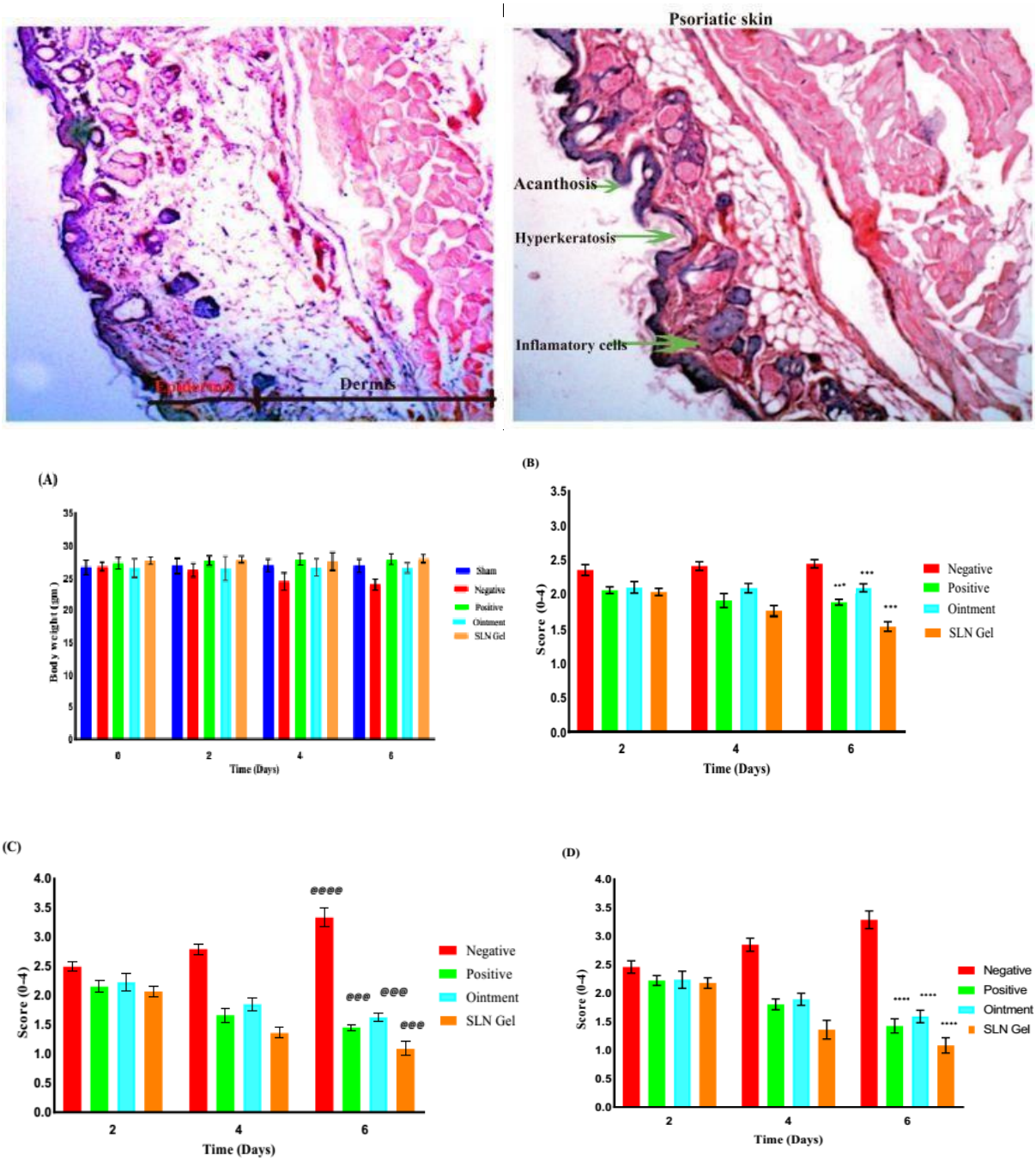
Figure 3. In vitro drug release profile from CP-SLNs gel, ointment and solution in phosphate buffer (pH 7.4). Values are expressed as mean ± SD (n = 3).

Anti-psoriatic efficacy in BALB/c mice IMQ induced psoriasis plaque model

A murine model of human psoriasis was developed in this study to investigate the efficacy of topical CP-SLNs carriers in psoriasis. IMQ-induced psoriasis-like inflammation acts as a model for the analysis of pathogenic mechanisms in psoriasis. Topical application of IMQ on mouse skin leads to the rapid proliferation of dendritic cells and keratinocytes to increase cytokine production. These effects in the mouse skin closely resemble human plaque- type psoriasis with respect to erythema, skin thickening, scaling, epidermal alteration (acanthosis, parakeratosis**)** Figure 4. From 3rd day after the IMQ application, the back skin of IMQ-treated mice began to display psoriatic features i.e., erythema, thickening, and scales. Phenotypical changes of mouse skin after sixth day of treatment with respect to formulation are shown in Figure 6. Observations were made in comparison with the negative control, positive control, Clobetasol ointment, Clobetasol-loaded SLNs gel, and efficacy of these

groups was compared with the sham group. Ear thickness was measured and the difference between the right and left ear thickness was interpreted. Negative control showed significant difference between right ear and left ear thickness among the treatment groups, no difference was observed in other treatment groups shown in (Figure 5(E).

Figure 4. Histopathological images of normal mice skin and imiquimod induced psoriatic mice skin.



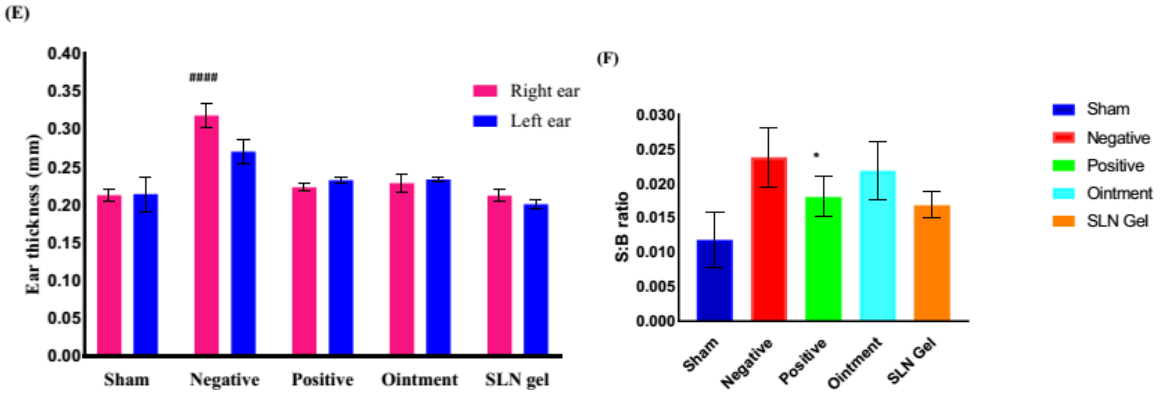


Figure 5. Efficacy of Clobetasol loaded SLN gel on IMQ induced psoriatic plaque model. BALB/c mice were treated daily with IMQ cream on the shaved skin and right ear for six consecutive days. Application of different treatments on their respective animal groups was started on the third day of IMQ application and erythema, scaling, and skin thickening was recorded on 0, second, fourth, and sixth day on a scale from 0 to 4. (A) Change in body weight; (B) PASI scoring for erythema; (C) PASI scoring for scaling; (D) PASI scoring for skin thickening; (E) ear thickness; where ####p<.001 (vs. thickness of left ear), #p<.008 (vs. Thickness of the left ear); (F) spleen weight to body weight ratio for different study groups.###p<.001 (vs. normal group), **p<.01 (vs. IMQ group), ***p<.001 (vs. IMQ group), **** p<.0001 (vs. IMQ group), @p<..0001 (vs. positive control group).

Body weight and PASI scoring

Body weight of all the groups was measured during the treatment period of sixth day. Slight increase in body weights was observed in positive, ointment, CP-SLNs and negative control showed slight decrease in body weights was observed except Sham group showed a constant increase in body weight from 0th day to 6th day Figure 5(A). To evaluate the efficacy of the formulation PASI scoring is one of the measurements. It is done on the scale of 0 - 4 on 0, 2, 4, 6 day and plotted (Figure 6 (B) (C) (D).

Erythema represents the degree of vasodilation due to release of cytokines (IL-1 and TNF-a) and other compounds like NO, phospholipase A2 metabolite, histamine in dermis from dendritic cells, keratinocytes, mast cells, etc. Increase in skin thickness refers to proliferation of keratinocytes due to pro-inflammatory cytokines (IL-20 and IL-22). Scaling reflects the poor differentiation of keratinocytes. Negative control exhibits erythema, scaling and thickness with score of 2-3 (severe) on sixth day of IMQ treatment which indicates the inflammatory responses were developed as compared to sham control group (Van der Fits et al., 2009). In comparison to negative control group, positive control group, ointment, and CP-SLNs gel showed reduction in with significant difference. Significant reduction in skin thickness and scaling was observed in positive control, ointment and CP-SLNs gel group in comparison with negative control.

Spleen enlargement is an important marker of immunological disorder. IMQ induces splenomegaly by increasing number of Th17 cells. Spleen weight to body ratio (SBWR) for all

study groups was calculated on seventh day of experiment. Increase in SBWR was observed in negative control group which indicates induction of psoriasis after application of IMQ. Other treatment groups resulted in the reduction of SBWR with no significant difference between them.

Histology

Histopathology images of skin samples were collected from mice and its representation of each group were shown in **(**Figure 6). Sham represents histology of normal skin, while negative control exhibited hyperkeratosis (thickening of stratum corneum) and parakeratosis (retention of nuclei in stratum corneum), acanthosis (proliferation of the epidermis) and discreet chronic inflammatory infiltrates in the dermis. Positive control group showed reduction in thickness of epidermis in comparison to negative control group. Clobetasol-loaded SLNs gel and Clobetasol ointment treated group showed very similar histopathological results to that of the normal group where epidermis was normalized and less infiltrates were observed. The histopathological results are in agreement with phenotypic images taken from each group of animals.

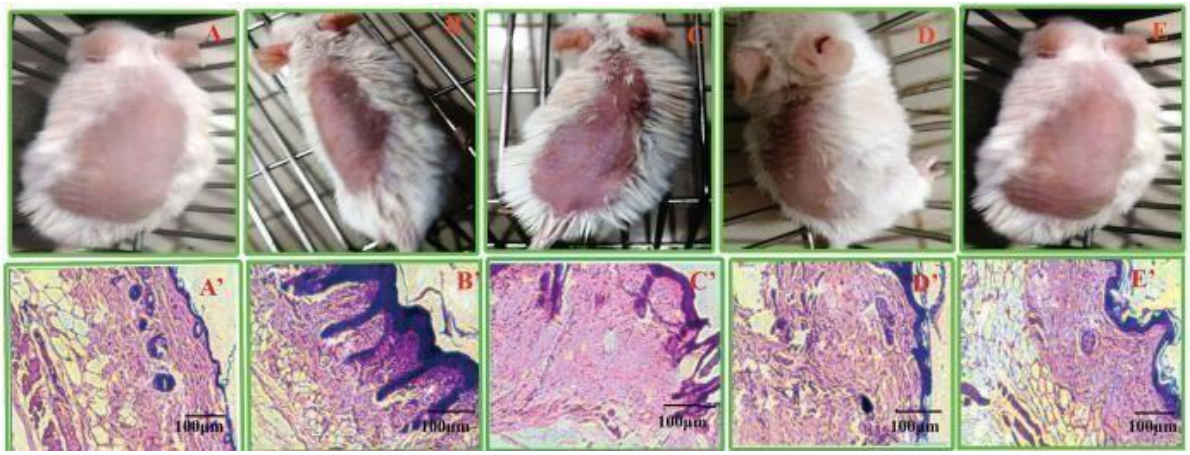


Figure 6. Phenotypic and histopathological features of skin during efficacy studies on IMQ induced psoriatic plaque model. (A and A') Normal group (arrow head and double-headed arrow indicate epidermis and dermis respectively), (B and B') negative control group, (C and C') positive control group, (D and D') ointment group, and (E and E') SLNs gel group. Each image is representative of respective group (n = 5).

Enzyme-linked immunosorbent assay

Psoriasis is a Th1/Th17 mediated inflammatory process associated with overexpression of Th1 and Th17-associated cytokines (IL-17, IL-22, IL-23, TNF- α) leading to inflammation and keratinocyte hyper proliferation. 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).

In this study, the efficacy of topically applied CP-SLNs gel was evaluated by determining the levels of IL-17, IL-22, IL-23, and TNF-a cytokines present in the skin homogenate collected on the seventh day of study as these cytokines play important role in psoriasis. Compared to sham group, negative control exhibited significant elevation of cytokine levels of IL-17, IL-22, IL-23, and TNF-a. In this efficacy study, CP-loaded SLNs gel showed 49.1, 66.9, and 69.6% reduction in IL-17, IL-22, and IL-23 levels, respectively, whereas Clobetasol ointment showed 38.1, 47.9, and 43.5% reduction in IL-17, IL-22, and IL-23 levels, respectively, compared to negative group. Positive control group which was treated with betamethasone showed 36.8, 71.1, and 53.1% reduction in IL- 17, IL-22, and IL-23 levels. TNF-a levels were reduced in CP SLNs group, ointment and positive control group by 47.8, 39.2, and

52.8%, respectively (Figure 7). Treatment with the CP-SLNs gel decreased the cytokines levels which confirmed the efficacy of formulation in treating psoriasis.

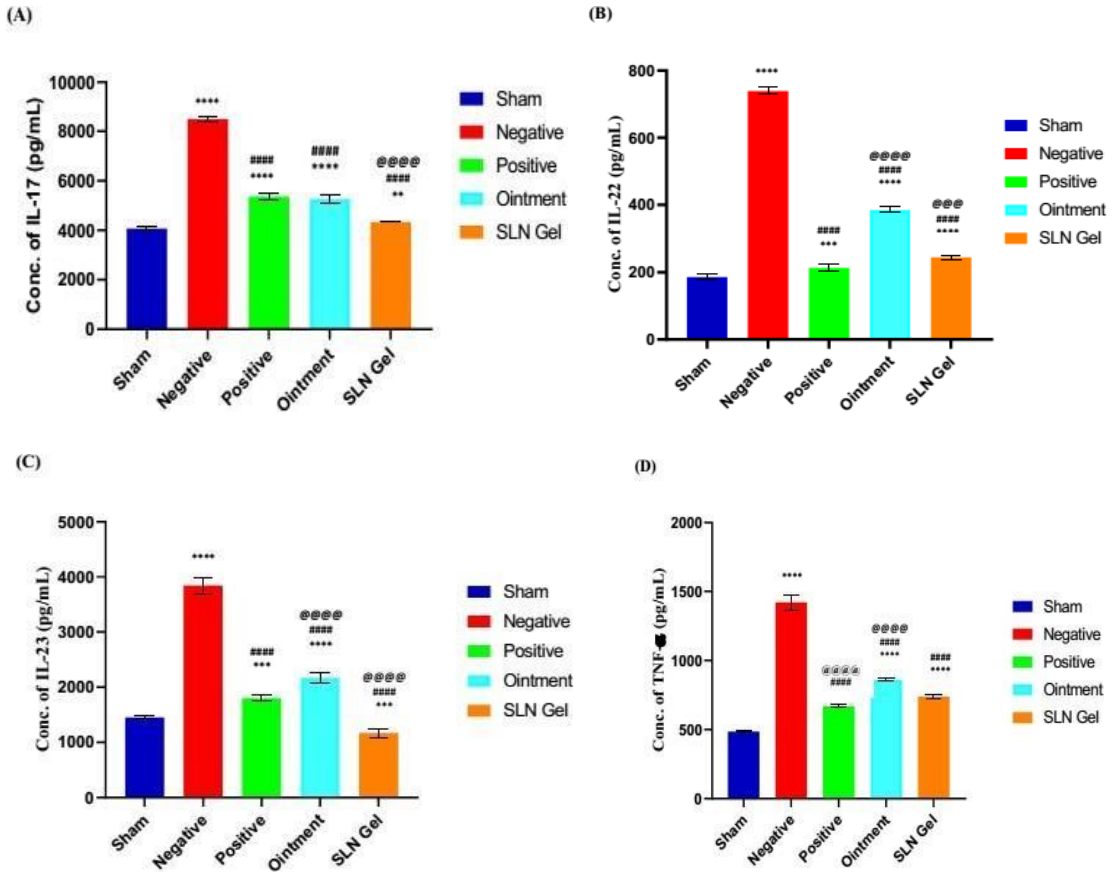


Figure 7. Determination of cytokine levels in skin homogenate after treatment by ELISA. ELISA experiments were performed as per manufactures protocol. (A) Levels of IL-17; (B) Levels of IL-22; (C) Levels of IL-17; (D) Levels of TNF-α. Values are expressed as mean ± SD, n=5. **p<0.0098(vs. Sham), ***p<0.0008(vs. Sham), ****p<0.0001(vs. Sham), $^{\#\#\#\#}$p<0.0001(vs. negative), $^{@@}$p<0.0001(vs. Positive), $^{@@@}$p<0.0001(vs. Positive), $^{@@@@}$p<0.0001 (vs. Positive).

4. CONCLUSION

Solid lipid nanocarriers of clobetasol were developed with an objective of enhancing the skin penetration of Clobetasol and thereby improving the efficacy of topical treatment psoriasis. Emulsification-homogenisation method was selected to prepare CP-loaded SLNs and the obtained Particle size, polydispersity index ,% entrapment efficiency and zeta potential obtained from this method were 133.3±3.66 nm, 0.179±0.081, 78.1±1.11% and -36.2±0.11 mV respectively. In vitro release profile suggested the sustained release of CP for prolonged time which would be beneficial in avoiding frequent administration of CP. CP-SLNs gel system provided ease of application, deeper penetration and slow release of drug. Topical application of the SLN gel showed reduction in psoriatic symptoms in IMQ induced psoriatic plaque model. Efficacy of SLN gel and ointment were compared the results like PASI scoring, histopathological study and ELISA showed that CP-SLNs gel have potential to treat psoriasis. To conclude SLNs gel was found to be more effective than ointment in treating psoriasis.

Authors Contributions
All the authors have contributed equally.

Conflict of Interests
The authors confirm no conflict of interest for this manuscript.

Ethical Approval
All experimental procedures were performed in accordance with the ethical guidelines for the study and was 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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