RESEARCH ARTICLE

Design and Characterization of Calcipotriol Proniosomal Gel: *In-vivo* Exploration against Imiquimod-induced Psoriasis in Experimental Animals

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ABSTRACT

The present work focuses on the preparation and evaluation of calcipotriol proniosomal gel (CPG) and find out if the antipsoriatic formulation could prevent imiquimod (IMQ) induced psoriasis in experimental animals. CPG was designed and developed by the coacervation phase separation method. Cholesterol and soya lecithin was used as membrane modifiers and tween 80 and span 60 as non-ionic surfactants. The developed proniosomal gels were characterized which revealed that the optimized batch CPG3 presented excellent formulation characteristics like viscosity of 9690 ± 1.43 cps, rate of spontaneity as 14 ± 1.37, %entrapment efficiency of 82.71 ± 0.48, the average hydrodynamic diameter of niosomes as 739.1 nm, polydispersibility index as 0.360 and ZP of -2.8 mV. *In-vivo* animal study of the optimized batch CPG3 was performed along with biochemical estimations of oxidative parameters such as glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA) and catalase; also, tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) were estimated using enzyme-linked immunoassay (ELISA) sandwiched. Histopathological estimation along with psoriasis area severity index (PASI) scores in the IMQ-induced psoriatic model revealed a significant decline in the scoring after treatment with CPG3 compared with a psoriatic group. Primary dermal irritation scoring was also evaluated on Sprague Dawley rats. The results revealed a considerable decrease in skin irritation score compared with a psoriatic group. Also, the levels of GSH, SOD and catalase were raised in CPG3 treated group as compared with a psoriatic group. While MDA level was declined after topical treatment with CPG3 compared with a psoriatic group. In conclusion, the optimized CPG3 showed promising pharmacological effects in the treatment of psoriasis.

Keywords: Calcipotriol proniosomal gel, Psoriasis, *In-vivo* exploration, Superoxide dismutase, Glutathione, Malondialdehyde.

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INTRODUCTION

Psoriasis is a type of immune-mediated disease in which skin cells multiply up to ten times faster than normal.[1] The disease with no known cause or cure affects people of all ages but is more frequently identified in adults than in children.[2] Treatment of psoriasis poses multiple therapeutic challenges to physicians because it not only affects the dermal tissues but is also associated with various comorbidities like psoriatic arthritis, inflammatory bowel disease, Crohn's disease, cardiovascular diseases and depression.[3] The systemic therapies of psoriasis are mostly associated with significant adverse effects. However, in the last couple of decades, topical drug delivery has emerged as a promising and efficient system for the treatment of psoriasis due to its site-specific action and ability to overcome systemic adverse effects.[4] Transdermal systems bypass first-pass hepatic metabolism increasing systemic bioavailability of the drug significantly.[5] It also contributes to better patient compliance by reducing dosing frequency due to its sustained release action.[6]

Methotrexate and cyclosporine along with some other medicines are the first choice of drugs for the systemic treatment of psoriasis.[7] These drugs can control psoriasis but they have some serious side effects. That's why there is a need of alternative topical medicines as well as less toxic compounds for overcome of psoriasis.[8] There is no proper treatment for the psoriasis. It is a lifelong disorder that particularly affects the mental and social lifestyle of the patient.[9] The given treatment only manages and controls the psoriatic symptoms.

A vitamin D3 analogue calcipotriol which is also known as calcipotriene is used topically for psoriasis.[10] It mainly declines

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epidermal cell proliferation and cell differentiation is promoted. It competes with the natural form of vitamin D for vitamin D receptors in regulating cell proliferation and differentiation.[10,11] It reverses abnormal keratinocyte changes in psoriatic skin and induces differentiation and also suppresses the proliferation of keratinocytes that ultimately normalize epidermal growth.[12] The common side effects associated with topical use of calcipotriol are skin irritation, skin rash and itching.[13-15] Severe side effects like skin burning, stinging, rash, aggravation of skin condition, increase in calcium levels, excessive thirst, frequent urination, tiredness, confusion, nausea, vomiting, constipation, anorexia, and loss of weight which may call for stopping the topical use of calcipotriol. Better stability and higher entrapment efficiency make proniosomal gel unique in formulation compared to other vesicular drug delivery systems. Entrapment of calcipotriol in vesicular form may avoid direct skin contact and reduce skin irritant effect.

MATERIALS AND METHOD

Antipsoriatic calcipotriol was purchased from Innovative Chemical Interchange Pvt Ltd, Hyderabad. Cholesterol and soya lecithin were procured from Sigma Aldrich, India. Surfactants tween 80 and span 60 were procured from BASF, Mumbai and Oxford Laboratory, Mumbai, respectively. Other excipients required for the formulation of proniosomal gel were of analytical grade.

Preparation of Calcipotriol Proniosomal Gel

Proniosomal gel was prepared by coacervation phase separation method. First of all, calcipotriol was dissolved in chloroform in a glass vial. Then add surfactants tween 80: span 60 (1:1), cholesterol and soya lecithin in the vial. The vial was heated on a magnetic stirrer with hot plate at (55–60ºC) at 40 rpm for 5 to 10 minutes to completely solubilize all ingredients. Phosphate buffer of pH 7.4 was heated to (55–60ºC) and added to the previously formed solution until a yellowish translucent proniosomal gel is formed. After formulation of proniosomal gel it was incorporated in 1.5% carbopol 934 base. The prepared proniosomal gel formulations (CPG1–CPG8) were kept in dark room for further use and characterisation.[16] calcipotriol proniosomal gel (CPG) was prepared by applying factorial design 2^3 as shown in Table 1.

Table 1: Composition of calcipotriol loaded proniosomal gels using 2^3 factorial design

Batch	*Calcipotriol (µg)*	*Cholesterol*	*Soya lecithin*	*Tween 80 : span 60 (1:1)*
CPG1	50	+	+	+
CPG2	50	+	+	-
CPG3	50	+	-	+
CPG4	50	+	-	-
CPG5	50	-	+	+
CPG6	50	-	+	-
CPG7	50	-	-	+
CPG8	50	-	-	-

Characterization of CPG

Viscosity

In this test, 10 g of the formulation was transferred to a glass vessel and the spindle of the viscometer was placed in the formulation. Later viscosity was measured by running the spindle at various revolutions such as 0.5, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 100 rpm with increasing and decreasing rpm and the average value was determined.[17]

Spontaneity analysis

The stipulated quantity of proniosomal gel (20 mg) was placed in a beaker container and was smeared uniformly on the walls of a beaker. Then, add 2 mL of normal saline solution to the beaker wall and keep aside for approximately 20 minutes. Then, the vesicles (niosomes) number was counted by Neubauer's chamber by placing a drop of the solution.[18]

Vesicle particle size and zeta potential measurement

The niosomes vesicle particle size (PS) and zeta potential (ZP) of the optimized CPG3 was determined with the help of Litesizer 500 ZP analyzer at 25ºC. First of all, take 100 mg of CPG3 and disperse it in 10 mL of phosphate buffer with mechanical shaking for 2 minutes.[19]

Entrapment efficiency

To determine entrapment efficiency 1-gm of CPG was dispersed in distilled water. The mixture was warmed and allowed to hydrated to form niosomes. Later the dispersion was centrifuged at 25000 rpm for 30 minutes. The upper clear liquid was evaluated by high-performance liquid chromatography (HPCL) to determine free drug concentration.[20]

Entrapment efficiency (%) = [(concentration of total drug - concentration of free drug)/ concentration of total drug] ×100

Animal Studies

Animals

Nulliparous Swiss albino mice and Sprague Dawley rats were used for this experimental examination. Standard laboratory conditions were provided to the experimental Animals for improve the results we provided 12/12 hours light-dark cycle, an atmosphere maintained under the humidity of 50 ± 5%, and room temperature (25 ± 2°C). Normal water *ad libitum* and normal pelletized feed diet is provided to animals (Amrut rodent Feed, Pune). The study was approved by the Animal Ethics Committee, Trans-Genica Services Pvt. Ltd. Nagardeola, Tal- Pachora Dist- Jalgaon, Maharashtra, India. (Approval No. 1277/PO/RcBt/S/09/CPCSEA/TRS/PT/023/000)

Imiquimod induced psoriatic model

To assess the anti-psoriatic activity of CPG3 against Imiquimod-induced psoriasis. We used Nulliparous Swiss albino mice (n = 6). Prior to study, mice were shaved from the backside. The experimental animals were divided as follows.
Group I: normal control.
Group 2: Psoriatic control

Group 3: Treatment 1 group/Plain proniosomal gel group (PPG)
Group 4: Treatment 2 group/Calcipotriol proniosomal group
Group 5: Standard group/ salicylic acid treated group
Treatment of CPG and PPG was initiated from 7th day to the end of the study, PASI score was noted.

None (0), mild (1), moderate (2), and severe (3) and very severe (4).

At the end of the study (14th day), all the animals were sacrificed and further investigations like biochemical, proinflammatory cytokines and histopathology were performed.[21]

- *Skin irritation studies (Primary dermal test)*

Animals were divided into 2 groups and study was performed to examine the potentiality of CPG3. The skin of animals was shaved and CPG3 along with 0.8% v/v formalin was applied on left of shaved back skin while right backside was considered as control. The animals were specially examined for erythema, and edema for 30 minutes and later on signs of erythema and edema was checked.[22]

- *Assessment of pro-inflammatory cytokines*

Cytokines like TNF-α as well as IL-6 was evaluated by using the skin homogenate sample with the help of using enzyme-linked immunoassay (ELISA) sandwich and finally, the concentration was estimated by using standard curve graph.[22]

- *Assessment of antioxidant parameters in skin*

Antioxidants like superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA) was estimated with the help of kits that are commercially available in the market.[22]

- *Histology*

At the end of the experimental protocol all animals were sacrificed and the shaved back skin sample of mice was collected, and stored in natural phosphate buffer and formalin. Followed by the sample was fixed, dehydrated, and finally fixed into paraffin. Finally, skin sections of 5 mm thickness were taken by using a microtome. Later on, sections were stained with hematoxylin and eosin. Furthermore, those sections were examined under the light microscope.

Table 2: Viscosity, rate of spontaneity and entrapment efficiency determination of different batches

Batch	*Viscosity (cps)*	*Rate of spontaneity*	*Entrapment efficiency*
CPG 1	9735 ± 1.56	12 ± 1.81	76.10 ± 0.37
CPG 2	9337 ± 1.87	11 ± 1.29	74.33 ± 0.70
CPG 3	9690 ± 1.43	14 ± 1.37	82.71 ± 0.48
CPG 4	8718 ± 1.43	10 ± 0.89	73.18 ± 0.90
CPG 5	8592 ± 0.99	10 ± 1.56	70.09 ± 0.46
CPG6	8615 ± 1.27	11 ± 1.91	69.32 ± 0.81
CPG7	8383 ± 1.44	9 ± 1.11	66.58 ± 0.69
CPG8	8467 ± 1.71	9 ± 1.75	67.91 ± 0.42

- *Statistical analysis*

The study was analyzed by one-way and two-way ANOVA using the software Graph Pad Prism 6.0, USA. Analysis is represented as mean ± SEM for TWO separate experiments with *p-value* < 0.001.

RESULTS AND DISCUSSION

Evaluation of CPG

Viscosity

In gel formulation, viscosity plays an important role because it is responsible for the spreadability, extrudability and release of the drug from the applied preparation. If gels are highly viscous it decreases the drug release by retaining the drug in the gel base and opposite to that if the viscosity of the gel is lower it increases the gel spreadability and release rate of the incorporated drug. The viscosity of the prepared CPG formulations was found from 8383 ± 1.44 to 9735 ± 1.56 cps (Table 2).

Spontaneity analysis

The rate of spontaneity is defined as a number of niosomes formed after hydration of proniosomal gel for 15 minutes. It quantitatively indicates the number of niosomes formed after the hydration of proniosomal gel. The results of rate of the spontaneity of CPG is excellent and presented results ranging from 9 ± 1.11 to 14 ± 1.37 (Table 2).

Entrapment efficacy

Drug entrapment efficiency is one of the core parameters used in the optimization of the prepared formulations. The more the drug entrapped in the vesicles more amount of drug will diffuse at the site of application. The results of drug entrapment efficiency of CPG presented satisfactory entrapment efficiency ranging from 66.58 ± 0.69 ± 0.25 to 82.71 ± 0.48 (Table 2).

Vesicle particle size and zeta potential measurement

The optimized batch CPG3 was characterized for PS and ZP. The graph of zeta sizer showed that the niosomes ranging from approximately 100 to 900 nm can be seen on the graph with an average hydrodynamic diameter of 739.1 nm. The polydispersibility index was found to be 0.360. The zeta potential of the optimized batch CPG3 was found to be -2.8 mV as shown in Figure 1.

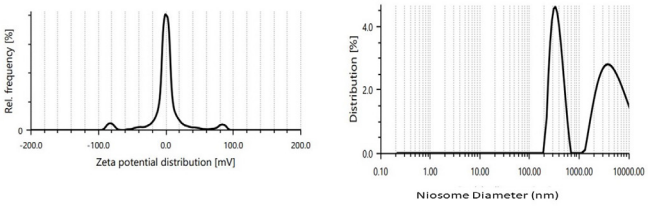

Figure 1: A- Zeta potential distribution, B- Niosomes size distribution – Intensity

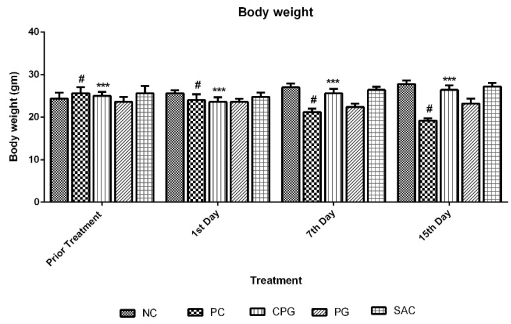

Figure 2: Effect of CPG3 on body weight in imiquimod induced psoriatic mice

Effect of CPG3 on body weight in imiquimod-induced psoriatic mice

In the current study, we found that there is a continuous reduction in the body weight of animals in a psoriatic group while the animals treated with CPG3 showed significant restoration of body weight as compared with psoriatic groups ($p < 0.001$) shown in Figure 2.

Effect of CPG3 on oxidative stress in imiquimod induced psoriatic mice

The level of antioxidants like GSH, SOD, and catalase were found to be decreased in a psoriatic group while the animals treated with CPG3 shows significant elevation in the level of those antioxidants as compared to the psoriatic group ($p < 0.001$). Also, the level of MDA is elevated in psoriatic animals and significantly reduced after treatment of CPG3 as shown in Figure 3.

Effect of CPG3 on proinflammatory cytokines in imiquimod induced psoriatic mice

Cytokines estimation was assessed by using ELISA sandwich assay. In animals in a psoriatic group it is observed that there is significant elevation of TNF- α as well as IL-6. That is normalized by the animals treated with CPG3. Figure 4

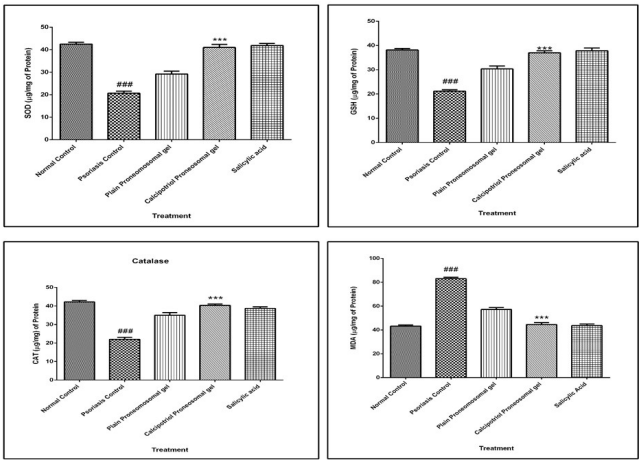

Figure 3: Effect of CPG3 on oxidative stress in imiquimod induced psoriatic mice

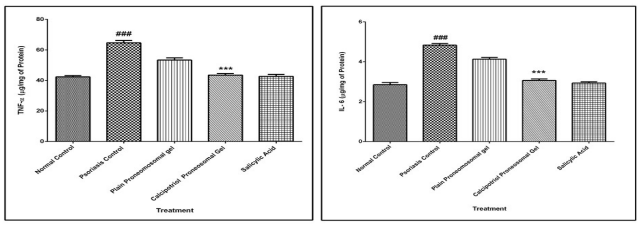

Figure 4: Effect of CPG3 on proinflammatory cytokines in imiquimod induced psoriatic mice

indicates that CPG3 have the potential to overcome psoriasis ($p > 0.001$).

Effect of CPG3 on PASI in Imiquimod induced psoriatic rat

PASI score was analysed in animals from 1st day to 7th day of the study. Remarkable elevation in PASI score was observed in psoriatic animals. Figure 5A shows that rats treated with CPG3 indicates a reduction in PASI score from 1st day to end of the study.

Effect of CPG3 on primary dermal irritation (skin irritation) in imiquimod induced psoriatic rat

Skin irritation index was analysed in animals from the 1st to 7th days. It was found that in psoriatic animals there was an elevation in skin irritation score. Figure 5B displays CPG3 treated animals have a consistent reduction in skin irritation score from the 1st to 7th days.

Effect of CPG3 on histopathology of the inflamed skin of mice in imiquimod-induced psoriatic mice

The psoriatic control group showed abnormal skin cell appearance and architecture. It was also observed that the infiltration of the skin cells with edema was increased. Figure 6 shows the mice treated with CPG3 showed recovery with normal skin cell appearance and architecture as observed by a reduction in spaces along with infiltration of skin cells.

The formulation variables can significantly influence the characteristics and the performance of the prepared formulation. The prepared proniosomal formulations were optimized on the basis of its viscosity, rate of spontaneity and entrapment efficiency. initially, different grades of non-ionic surfactants like tween and span were screened. On basis of

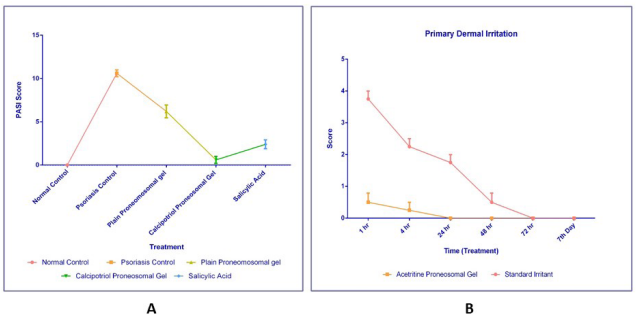

Figure 5: A - Effect of CPG3 on PASI in imiquimod induced psoriatic Rat, B -Effect of CPG3 on primary dermal irritation (Skin irritation) in imiquimod induced psoriatic rat

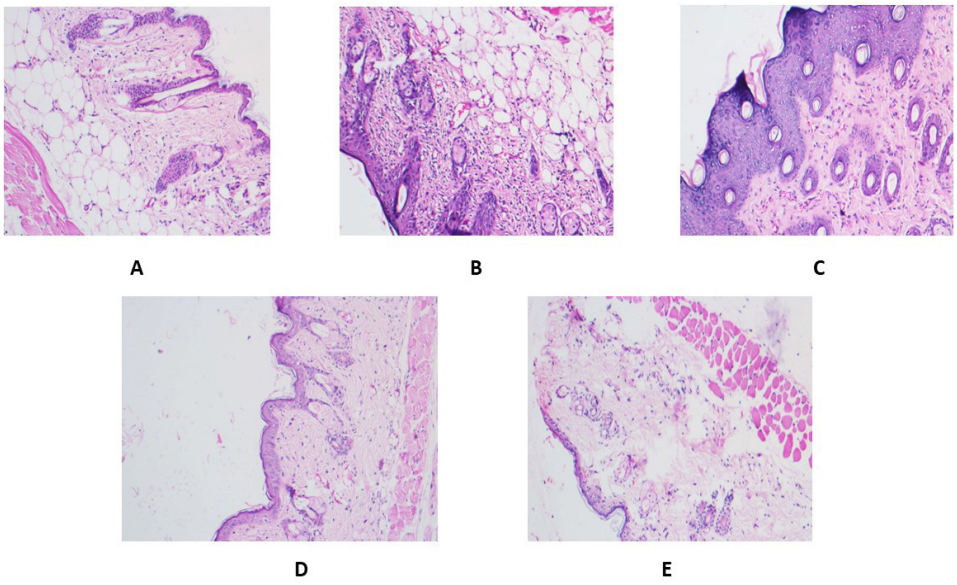

Figure 6: Effect CPG3 on histopathology of the inflamed skin of mice in imiquimod induced psoriatic mice. A. Normal Control; B. Psoriasis Control; C. Plain Gel; D. CPG3; E. Salicylic acid. Scale bar =100 µ

pre-formulation studies and its results the selected surfactants i.e., tween 80 and span 60 (1:1), lecithin and cholesterol were varied thus at high levels and low levels. At last, the drug concentration was optimized. Several shreds of evidence suggested that viscosity was higher with higher concentrations of cholesterol and surfactants, tween 80 and span 60 contrary to that viscosity decreased at lower concentrations of cholesterol and surfactants.[23-25] Entrapment efficiency (EE%) of the developed proniosomal gels with two surfactants tween 80 and span 60 in ratio of 1:1 revealed the highest EE% of value 82.71 ± 0.48 and lowest EE% of 66.58 ± 0.69. On the basis of earlier reported literature higher concentrations of cholesterol and surfactants tween 80 and span 60 the rate drug entrapment was higher whereas at lower concentrations it was decreased.[26,27] Additionally, psoriasis is characterized by the release several cytokines such as interferon- γ, TNF-α and the number of interleukins involved in the inflammation are followed by infiltration of immune cells into the skin and finally hyperkeratosis. *In-vivo* antipsoriatic activity was evaluated on basis of PASI scorings along with primary dermal irritation study. A higher degree of erythema and thickening of the back skin and scales were significantly seen in the psoriatic group whereas CPG3-treated group showed a remarkable recovery. From the first day to end of the study (14^{th} day), it was observed that CPG3 showed a remarkable reduction in skin thickness and PASI score. Furthermore, from 7^{th} day to 14^{th} day, the CPG3 found to have a considerable protective effect. Also, in histopathological screening, it was found that the IMQ-induced parakeratosis, acanthosis, epidermal cuticle and perivascular infiltration of inflammatory cells were normalized by the treatment of CPG 3. In summary, calcipotriol-loaded proniosomal gel presented excellent pharmacological effect against IMQ-induced psoriasis. This finding suggests that calcipotriol-loaded proniosomal gel has the potential as a therapeutic agent to overcome psoriasis.

CONCLUSION

In this present research work, we developed calcipotriol-loaded proniosomal gel for the treatment of psoriasis. Characterization and evaluation revealed the stability of the formulation. The prepared calcipotriol proniosomal gel has a characteristic nano size and shows a significant fall in PASI scores after treatment in imiquimod-induced psoriasis mice; therefore, calcipotriol proniosomal gel can be used for effective treatment of psoriasis.

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