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Investigation of Tacrolimus Loaded Nanostructured Lipid Carriers for Topical Drug Delivery

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The objective of this investigation was to develop nanostructured lipid carriers (NLCs) of tacrolimus by the hot homogenization technique by sonication. NLCs are commonly prepared by emulsification and lyophilization. The feasibility of fabricating tacrolimus-loaded NLCs was successfully demonstrated in this study. The developed NLCs were characterized in terms of their particle size, zeta potential, entrapment efficiency (EE) of tacrolimus, and morphology. Studies were conducted to evaluate the effectiveness of the NLCs in improving the penetration rate through hairless mouse skin. Tacrolimus-loaded NLCs were found to have an average size of 123.4 ± 0.3 nm, a zeta potential of −24.3 ± 6.2 mV, and an EE of 50%. *In vitro* penetration tests revealed that the tacrolimus-loaded NLCs have a penetration rate that is 1.64 times that of the commercial tacrolimus ointment, Protopic®.

Key Words : Tacrolimus, Nanostructured lipid carriers, Topical drug delivery, Nanoparticles

Introduction

Tacrolimus is a strong immunosuppressive non-steroidal anti-inflammatory drug (NSAID) used for the management of organ transplant recipients and in acute conditions of inflammatory skin diseases such as atopic dermatitis (AD). Tacrolimus binds to an intracellular protein called the FK-506 binding protein (FKBP) and forms a complex together with calcium, calmodulin, and calcineurin; this complex inhibits calcineurin activity. This results in the suppression of T cell activation and also effects the activation of B cells.[1] Tacrolimus is used clinically either orally or topically under the commercial name Prograf® and Protopic®[2,3] in 2000, topical tacrolimus, Protopic®, was approved for clinical use as a drug that can be used to treat moderate to severe AD. The adverse effects of tacrolimus include transient burning, sensation, and itching. Recently, the potential cancer risk of the use of the Protopic® ointment was reported, and the use of Protopic® in pediatric patients less than 2 years old is avoided.[4,5]

In topically administered drugs, the penetration rate is very important factor to determine their dosage regimen. We considered that lowering the concentration can contribute to reducing the adverse events. In this regards, we try to develop new carrier system having better penetration profile, which could be the positive effects on clinical use of tacrolimus. The use of lipid nanoparticles in various pharmaceutical applications, especially in parenteral, peroral, and ocular applications, has been investigated; the use of lipid nanoparticles in dermal applications has also been investigated.[6] The first generation of lipid nanoparticles and solid lipid nanoparticles (SLNs) was developed at the beginning of the 1990s as an alternative carrier system to emulsions, liposomes, and polymeric nanoparticles.[7] Lipid nanoparticles have been studied as an alternative to other novel delivery systems because they offer various advantages; for example, they facilitate incorporation with lipophilic and hydrophilic drugs, have physical stability, are inexpensive, and are easier to manufacture than liposomes. Nanostructured lipid carriers (NLCs) are the second generation of lipid nanoparticles and were developed to overcome some limitations associated with SLNs. These particles are produced using blends of solid and liquid lipids. NLCs have a higher loading capacity for a number of active compounds, and the potential expulsion of active compounds during storage is minimized.[8,9]

In general, the drug can be deposited in between the fatty acid chains or in between the lipid layers; the drug can also be deposited in imperfections of the lipid matrix that can be controlled such that the drug penetrates and partitions into the skin.[10,11] Both NLCs and SLNs have many advantages in dermal applications. They are composed of biodegradable lipids exhibiting low toxicity and excellent tolerability after application. Because of the small size of NLCs, close contact with the stratum corneum is guaranteed and the amount of the drug penetrated into the skin can be increased.[12] Furthermore, lipid nanoparticles can enhance the chemical stability of components that are sensitive to light, oxidation, and hydrolysis.[13]

In this present study, we prepared NLCs containing tacrolimus successfully and proved the enhanced flux through the hairless mouse skin.

Experimental Section

Materials. Tacrolimus (FK-506) was obtained from Langfang Shinya Chemicals Co. Ltd. (Hongkong, China), glycerol monostearate(GMS), oleic acid and dimethyl glycol

monoethyl ether (DGME) was obtained from Daejung Chemicals & Materials Co. Ltd. (Seoul, Korea). Polysorbate 80 or Tween 80 was purchased from Sigma Chemicals Co. Ltd. (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade solvents were purchased from J. T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ). All other chemicals were reagent grade or chromatographic grade.

Preparation of NLCs and Formulation. NLCs composed of glycerol monostearate and dimethylglycol monoethyl ether were prepared by the hot sonication and homogenization technique. The process involved, dissolving 1 mg of tacrolimus in 30 mg dimethylglycol monomethyl ether and heating at 70 °C, which is 5 °C above the melting point of GMS. This solution was then added to molten GMS at 70 °C. The aqueous phase was prepared separately by dissolving 50 mg of Tween 80 in 1 mL of water. The organic solution was then quickly dispersed into the aqueous phase, and the mixed solution was stirred vigorously for 5 min forming a primary emulsion. The NLCs were finally prepared by homogenizing the primary emulsion using a probe sonicator for 2 min. The remaining surfactant was eliminated by ultra centrifugation at 18,000 rpm for 15 min. After centrifugation, the clear solution was decanted and the supernatant was redispersed in water. After this, the solution was freeze-dried at −70 °C. Blank NLCs without the drug in the lipid phase were prepared in the same manner. SLNs were made by only GMS as the lipid phase in the same manner of NLCs preparations.

Particle Size, Zeta Potential and Morphological Analysis. The size of NLCs was obtained using the dynamic light scattering technique by the Malvern Zetasizer 3000HAs system (Malvern Instruments, Worcestershire, UK) at 25 °C at an incident angle of 90° and analyzing the data with the PCS 1.61 software. For the measurements, freshly prepared particles were appropriately diluted in deionized water and each sample was measured in triplicate. The morphological examination of NLCs was carried out by field emission scanning electron microscope (FE SEM, JSM 840-A, Jeol Ltd., Japan). The freeze-dried NLCs were redispersed in deionized water for appropriate separation and a droplet of the resulting suspension was placed on an aluminum foil and coated with a 100 Å layer of platinum (Cressington 108, Jeol Ltd, Tokyo, Japan). The dimensions of the NLC particles in the SEM images were measured by comparison with the scale bar.

Encapsulation Efficiency. The amount of encapsulated tacrolimus was calculated by subtracting the free amount of the drug from freeze-dried NLCs. NLCs were dissolved in methanol and warmed to dissolve completely, and the residual lipid was separated by ultracentrifugation at 10,000 rpm for 5 min. The supernatant was collected and filtered through a PTFE syringe filter and analyzed using HPLC. Entrapment efficiency (EE%) was calculated from the following equation.

$$\text{Encaptulation efficiency (EE, \%)} = \frac{\text{Actual amount of drug loaded in NLCs}}{\text{Theory amount of drug loaded in NLCs}} \times 100$$

; Actual amount of drug in NLCs is amount ratio of drug in freeze dried NLCs. Theory amount of drug in NLCs is amount ratio of drug in total mixture of drug and lipid materials.

DSC Measurement. Differential scanning calorimetric (DSC) analysis was performed by a calorimeter (TA instrument, USA). To avoid the appearance of broad water peaks, the lipid material and the NLCs with or without tacrolimus, were freeze dried before the DSC measurement. The samples were then loaded onto the aluminum pan under nitrogen at a flow rate of 50 mL/min. The heat flux instrument was equipped with a refrigerated cooling system and was calibrated using a standard, while the sample was run against a hermetically sealed empty reference pan. Prior to heating, the sample was equilibrated at 25 °C for 10 min and evaluated between 25 and 100 °C with a heating or cooling rate of 5 °C/min. The analysis was repeated three times and expressed as the mean of three determinations.

HPLC Analysis. Tacrolimus was analyzed by reverse phase HPLC (Agilent 1100 series, Agilent, USA) by a C18 column (5 μm, 4.6 mm × 150 mm, Waters Inc.). The HPLC system consisted of a quaternary pump, an autosampler, UV detector and a workstation. The column temperature was set to 50 °C. The mobile phase was an acetonitrile-deionized water system at a ratio of 70:30 (v/v) under isotropic conditions with a flow rate of 1.1 mL/min. Tacrolimus eluted at 8 min under the conditions and was detected at 210 nm. No interference from the formulation or skin tissue was observed. All samples were filtered through a 0.22 mm pore size filter membrane before determination.

***In vitro* Permeation Study.** Six-week-old hairless mice were purchased from Orientbio Co. Ltd. (Seoul, Korea) and were maintained under specific pathogen free and conventional conditions respectively. All procedures were reviewed and approved by the Animal Care and Use Committee of Seoul National University. The full thickness of mouse skin was obtained after anesthetizing the mouse with Zoletil® and sacrificing them. The cutaneous fat and blood vessels were removed gently by washing with phosphate buffer solution (PBS) and the skin kept under −20 °C for up to 1 month for the experiments. *In vitro* percutaneous penetration was studied using static Franz type diffusion cells. The exposed skin area was 2.09 cm^2 and the volume of the receptor chamber was 10 mL. A mixed solution of phosphate buffered saline and ethanol (3:1 v/v) was used for the receptor medium, while 0.1% Protopic® was used for the control group. Tacrolimus-loaded NLCs and tacrolimus only were prepared at a 0.01% concentration using an emulsion that contained oleic acid, Tween 80, and deionized water. All samples used in the penetration test were freshly prepared and applied onto the skin immediately. 50 mg of topical sample was applied to the epicutaneous side of the skin in a donor chamber. 500 mL of sample was collected from the receptor medium at various time intervals for up to 24 h and replaced with fresh medium. All experiments were performed three times at 37 °C.

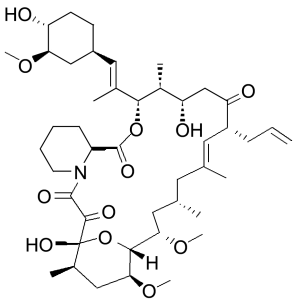

Figure 1. Structure of tacrolimus.

Results and Discussion

Characterization of Nanostructured Lipid Carriers. NLCs containing tacrolimus were developed by the homogenization method through sonication at high temperature (70 ºC). NLCs were prepared using GMS as the core material and DGME as the liquid matrix that helped in solubilizing tacrolimus. DGME belongs to the family of glycol ethers and is used as a solubilizing agent and an absorption enhancer.[14,15] Tween 80 is also widely used as an emulsifier or surfactant to make polymeric nanoparticles, lipid nanoparticles, emulsion, etc. The size, zeta potential, and EE of the prepared particles are shown in Table 1. As is evident, NLCs using both GMS and DGME are appropriate for topical delivery of tacrolimus. The particles in this formulation have a diameter of 123.4 ± 0.3 nm, EE of 50%, zeta potential of −24.3 ± 6.2 mV, and a drug content of 1.96%. Although Tween 80 is a non ionic emulsifier, the zeta potential of NLCs was negative. This could be due to the chemical structure and partial hydrolysis of tween 80, but the exact mechanism is unknown.[16,17] We also prepared SLNs with GMS to compare the characteristics of NLCs with those of SLNs. Both SLNs and NLCs without tacrolimus have similar properties, suggesting that tacrolimus did not significantly change the characteristics of NLCs. Finally the FE-SEM images in Figure 2 reveal the almost spherical but irregular shape of the particles in NLCs. In other reports, nonsperical shape of NLCs were reported, which is due to the lipid modification. This is not an impediment for dermal administrations.[18]

Thermal Analysis. DSC analysis was performed to determine the physical state of NLCs both in the presence and absence of tacrolimus. Figure 3 shows the DSC curve for GMS bulk powder and NLCs with or without tacrolimus. The mixture of GM and DGME showed a main melting peak at 61.90 °C. The NLC melted at a comparable temperature both in the presence and absence of the drug, at 61.05 °C in the absence of the drug and at 61.71 °C in the presence of the drug. It can therefore be suggested that the main lipid GMS entrapped in the NLC is solidified perfectly. Since the

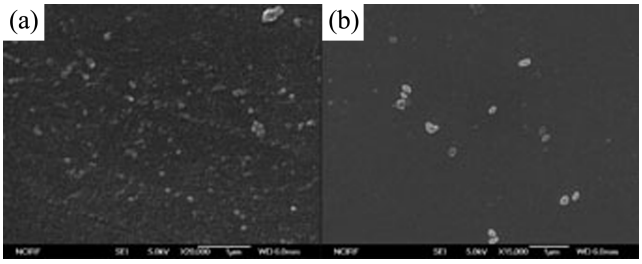

Figure 2. FE-SEM images of no drug loaded NLCs (A) and tacrolimus loaded NLCs (B).

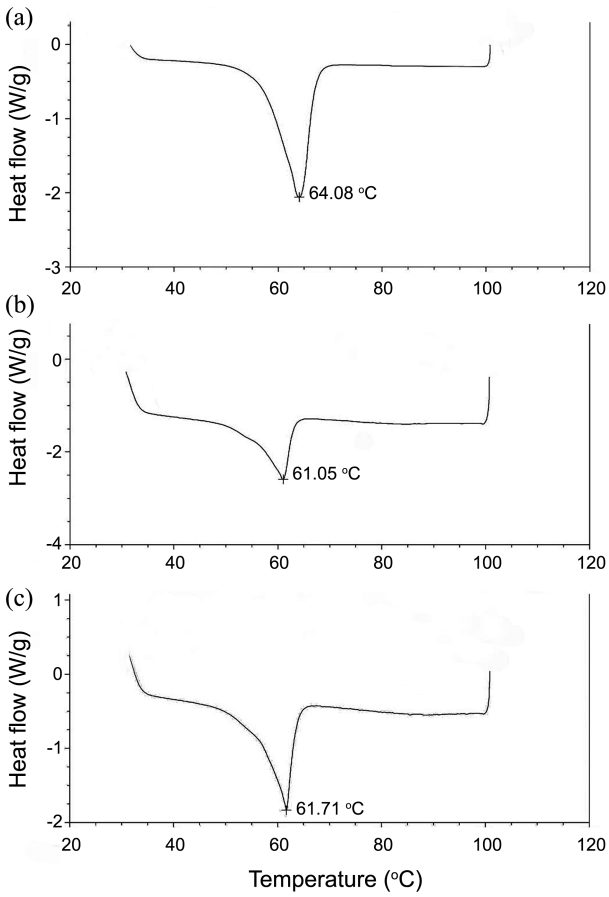

Figure 3. Differential scanning calorimetry of mixture of GMS and DGME (A), no drug loaded NLCs (B), and tacrolimus loaded NLCs (C).

Table 1. Formulation and characteristics (size, zeta potential, and encapsulation efficiency (EE)) of NLCs and SLNs (Asterisk means NLCs without drug)

	GMS (mg)	DGME (mg)	T80 (mg)	DW (mg)	Drug (mg)	Size (nm)	Zeta potential (mV)	EE (%)
SLNs	100	0	30	1000	5	126.7 ± 1.6	−27.5 ± 5.3	45
NLCs	100	30	30	1000	5	123.4 ± 0.3	−24.3 ± 6.2	50
NLCs*	100	30	30	1000	0	125.5 ± 1.4	−26.7 ± 2.8	–

melting point is higher than 40 °C, it is appropriate for topical delivery.

***In vitro* Permeation Test.** We investigated the effect of NLCs on the penetration of tacrolimus. It was reported that NLCs is better than SLNs in particle stability like drug expulsion and loading capacity.[19] In this reason, we choose the NLCs as carrier to delivery tacrolimus. Figure 4 shows the flux of tacrolimus across hairless mouse skin from NLCs mixed in an emulsion, tacrolimus loaded in an emulsion, and Protopic®. The plots show the cumulative amounts of tacrolimus permeated through the skin as a function of time. Protopic® showed a penetration rate of 5.4 ± 0.5 mg/cm^2 over a 24 h period. However, in case of the emulsion and NLCs mixed with emulsion, the total amount of penetrated tacrolimus was 5.0 ± 0.7 μg/cm^2 and 8.6 ± 0.1 μg/cm^2 respectively. We initially planned to mix NLCs into the formulation of Protopic® to improve the effect of the NLCs, but the formulation of Protopic® which uses paraffin base was very sticky and did not disperse NLCs. Therefore we prepared an emulsion type formulation that had a similar flux as Protopic®. As a result, tacrolimus incorporated emulsion had almost the same flux as that of Protopic®, while tacrolimus-loaded NLCs had an increasing penetration rate compared to the emulsion and Protopic®. The penetration rate for tacrolimus-loaded NLCs was found to be almost 1.64 times higher than either Protopic® or the emulsion formulation. We have investigated other types of formulation using isopropyl myristate/water, stearic acid/water, and hydrogel with 1% hydroxypropylmethyl cellulose, in which we have obtained similar penetration rates as that of Protopic® (data not shown). The high molecular weight and lipophilicity of tacrolimus prevents to pass the stratum corneum.[20] In general, NLCs do not penetrate the horny layer but a follicular uptake by the hair follicle has been reported in case of particulate systems.[21] The improved dermal uptake of active compounds loaded to NLCs might also result from an increased contact to the surface of the active compound with the corneocytes followed by either a rapid or steady release.[22,23] In Table 2, the amount of remaining tacrolimus is almost same in all the formulations, which means that all the formulations have similar abilities of partitioning into the dermal layers with different penetration rates through the hair follicles.

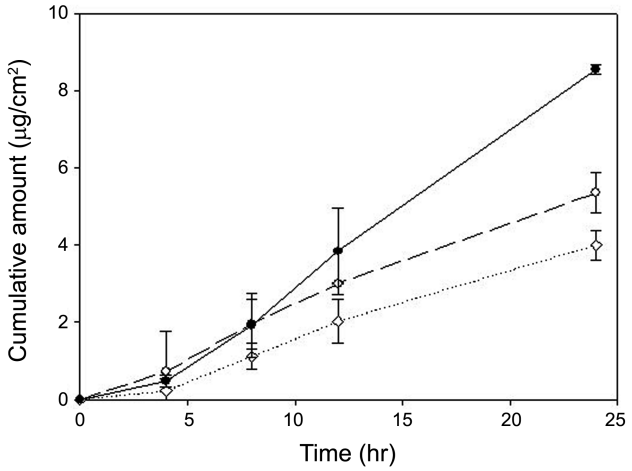

Figure 4. *In vitro* penetration test of Protopic® (○), Oleic acid Emulsion (◇), and NLCs in Oleic acid emulsion (●); the test was performed using Franz diffusion cell.

Table 2. Concentration of tacrolimus remaining on the skin after *in vitro* penetration test

Protopic® (μg/cm^2)	OA Emulsion (μg/cm^2)	NLCs_OA emulsion (μg/cm^2)
21.1 ± 3.2	21.2 ± 1.8	20.9 ± 4.1

Generally, Drug applied on skin may pass the skin through both the the stratum corneum and hair follicles. In case of using particles like NLCs, they allows reducing the trans epidermal pathway.[24] *In vivo* and *in vitro* studies demonstrated that topically applied particles make drug pass through the horney layers, in the lower follicular tracts, which is incomplete corneocytes. The corneocytes in this region are smaller and induce the faster penetration into the vialble membranes. In fact, there is little evidence that particles at a size exceeding 100 nm pass to the vialble epidermis of intact skin.[25] We have to investigate the exact penetration pathway of NLCs for further studies and develop an ointment base that would stabilize the NLCs for a longer period of time.

Since tacrolimus-loaded NLCs were observed to have enhanced penetration rate profile, a topical product of lower concentration can thus be developed with decreased adverse and high therapeutic effects.

Conclusions

In the present study, tacrolimus-loaded NLCs were successfully prepared by a simple sonication method. The penetration rate of these NLCs through the skin of a hairless mouse was greater than that of Protopic®, which is a commercial dermal ointment containing tacrolimus. These results suggest that tacrolimus-loaded NLCs have significant potential for use as an alternative topical formulation for tacrolimus.

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