Global Research Laboratory[1], Department of Biochemistry and Division of Brain Korea, College of Medicine, Korea University; Research Institute of Pharmaceutical Sciences[2], College of Pharmacy, Seoul National University, Seoul; Korea Science Academy[3], Busan; Department of Bioscience and Biotechnology[4], Sejong University, Seoul, Korea

Topical administration of cyclosporin A in a solid lipid nanoparticle formulation

Sung Tae Kim[1], Dong-Jin Jang[2], Jong Hyo Kim[3], Joo Yeon Park[3], Ji Soo Lim[3], So Yeon Lee[3], Kyung-Mi Lee[1], Soo-Jeong Lim[4], Chong-Kook Kim[2]

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Dr. Chong-Kook Kim, College of Pharmacy, Seoul National University, San 56-1, Shillim-dong, Kwanak-gu, Seoul, Korea 154–741
sungtae7@snu.ac.kr

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Cyclosporin A (CsA)-loaded solid lipid nanoparticles (SLN) were developed for improved skin penetration. CsA-loaded SLN, prepared using a hot homogenization method, were nano-sized (about 73 nm) with a slightly negative surface charge (about −16 mV) and stable under physiological conditions regardless of CsA incorporation. *In vitro* permeation studies using murine skin mounted in the Franz-type vertical diffusion assembly revealed that the skin permeation efficiency of CsA-loaded SLN was 2-fold higher than that of CsA-oil mixture in viable skin. Furthermore, topically administered CsA-loaded SLN relieved symptoms of atopic dermatitis (AD) in an *in vivo* murine model of AD by decreasing the T helper (Th) 2 cell-related cytokines interleukin (IL)-4 and -5. These results suggest that SLN are effective drug carriers for topical delivery and that CsA-loaded SLN can be therapeutically applied in allergy-related skin disorders.

1. Introduction

Cyclosporin A (CsA), a non-polar cyclic polypeptide that consists of 11 amino acids with a molecular weight of 1,203 Da, is a potent immunosuppressant that inhibits cell-mediated immunity, mainly through inhibition of T helper cells (Lee et al. 2004; Robson et al. 2003). CsA, which is practically insoluble in water, is widely used in the prevention of transplant rejection and the treatment of immunological disorders such as atopic dermatitis (AD) (Leung et al. 2004). Recently, CsA has been applied to the treatment of patient with atopic dermatitis (AD), which affects up to 20% of people in the United States, through the oral administration (Robson et al. 2003; Lee et al. 2004), furthermore, topical administration (Russell 2002).
Various formulations for administration of CsA have been reported including microemulsions, self-emulsifying drug delivery system (SEDDS), cyclodextrin microspheres, solid dispersions, and liposomes (Bekerman et al. 2004; Ugazio et al. 2002). Among them, a cyclosporine-loaded microemulsion filled in a soft gelatin capsule, Sandimmun®, is available commercially for oral administration, and a lipid-based formulation has been studied to enhance the oral bioavailability of CsA (Müller et al. 2006; Neslihan et al. 2004). Likewise, many efforts have been focused on developing CsA formulations with improved drug bioavailability after oral administration. However, systemic administration of CsA via the oral route may give rise to side effects, such as nephrotoxicity and a narrow therapeutic index, compared to topical administration (Verma et al. 2004). Therefore, attempts have recently been made to administer CsA via alternative routes other than the oral route to avoid this side effects (Verma et al. 2004; Tatlipinar et al. 2005). For example, CsA eye-drops have been safely used in ocular surface disorders such as vernal keratoconjunctivitis, atopic keratoconjunctivitis, and keratitis (Tatlipinar et al. 2005). Transdermal administration of CsA has previously been attempted by an electroporation technique, a lecithin-based vesicular carrier system, or a penetration enhancement system using ethanol and phospholipids (Verma et al. 2004; Wang et al. 1998; Guo et al. 2000). When topically administered via a transdermal route, however, CsA by itself generally cannot penetrate the skin barrier, particularly the stratum corneum (SC), since CsA is a molecule with a relatively large molecular weight (>350 Da) (Song et al. 2006; Karande et al. 2004).
Recently, solid lipid nanoparticles (SLN) have been widely studied as a next-generation delivery system in pharmaceuticals and cosmetics (Wissing and Müller 2002, 2003). Compared with other lipid-based carriers, SLN has several advantages such as enhanced stability of loaded drugs against chemical degradation, smaller particle size, and easier adhesiveness on the skin due to film formation (Müller et al. 2000; Lim et al. 2004). According to a previous report, SLN comes in close contact to the SC, thus increasing the amount of bioactives that penetrate viable skin (Mei et al. 2003). SLN also forms a coherent lipid film that can have an occlusive effect (Wissing and Müller 2002; Müller et al. 2000). These reported advantages of

Table: Size, zeta potential and entrapment efficiency of SLN and CsA-loaded SLN

	Size[a] (nm)	Zeta potential[a] (mV)	Entrapment efficiency[a] (%)
SLN	47.80 ± 1.65	−16.15 ± 1.90	–
CsA-loaded SLN	73.37 ± 0.67	−15.86 ± 2.88	90.80 ± 0.36

a. Data represents mean ± standard deviation (n = 3)

SLN as a topical delivery system prompted us to investigate the SLN formulation of CsA for its topical administration.

This study was undertaken to develop CsA-loaded SLN formulations and to evaluate its physical characteristics and its ability to penetrate the skin and, furthermore, to predict its therapeutic ability in atopic dermatitis.

2. Investigations and results

The physical properties and entrapment efficiency of empty- and CsA-loaded SLNs are shown in the Table. In size comparisons, CsA-loaded SLN was about 25 nm larger than empty SLN. The average zeta potentials of empty- and CsA-loaded SLN were similar (−16.15 ± 1.90 and −15.86 ± 2.88, respectively). These results demonstrate that CsA incorporation affects the mean particle size of SLN, but barely affects the zeta potential value. The entrapment efficiency of SLN was higher than 90% for CsA.

To investigate the physical stability of SLN, time-dependent changes in the particle sizes of empty- and CsA-loaded SLN were investigated. As shown in Fig. 1a, the particle size of CsA-loaded SLNs remained below 100 nm during storage, although the mean size increased slightly

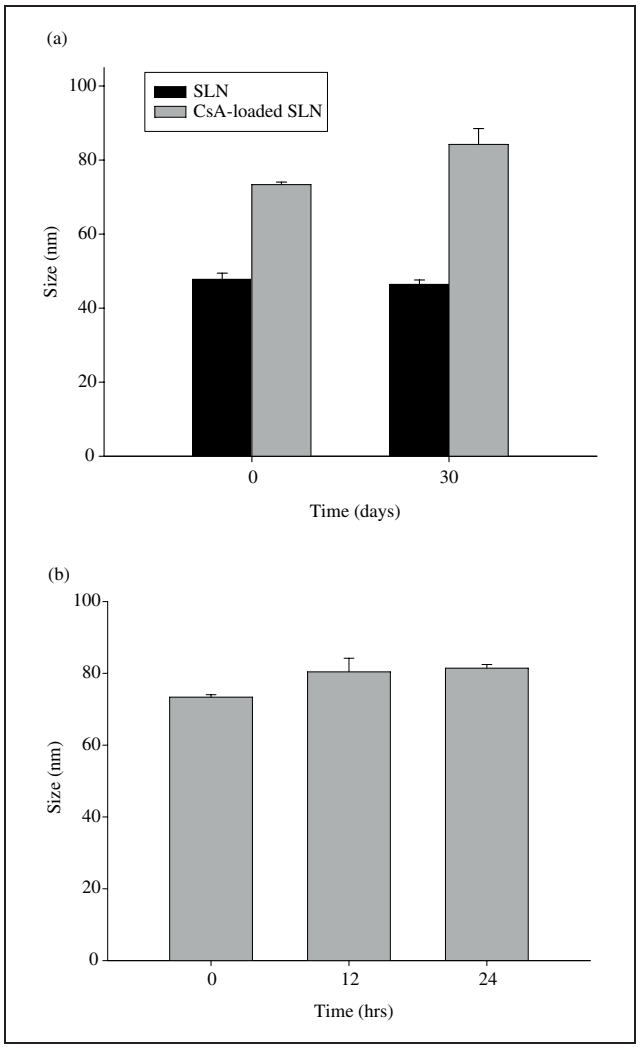

Fig. 1: Stability of SLN and CsA-loaded SLN. (a) The size of SLN and CsA-loaded SLN were determined after the storage at 4 °C (n = 3). (b) CsA-loaded SLN was stored in 10% serum media for 24 h. Data represents mean ± standard deviation (S.D.) (n = 3)

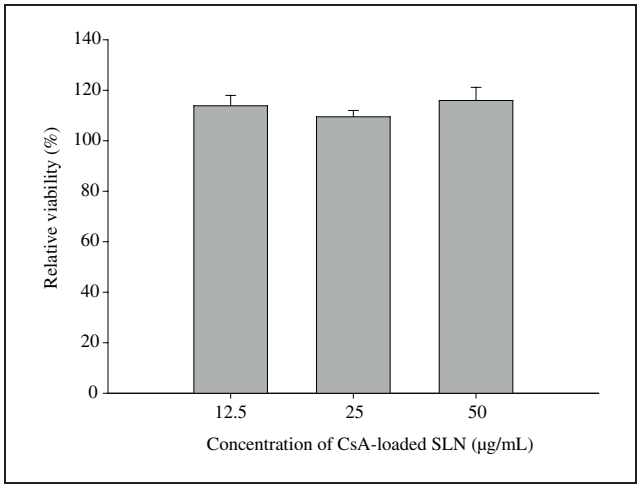

Fig. 2: Cytotoxicity of CsA-loaded SLN. After seeding D10.G4.1 cells onto a 96-well plate (1×10^4 cells/100 μl), up to 50 μg/mL CsA-loaded SLN was added to each well. Cell viability was analyzed by XTT assay. Data represents mean ± S.D. (n = 3)

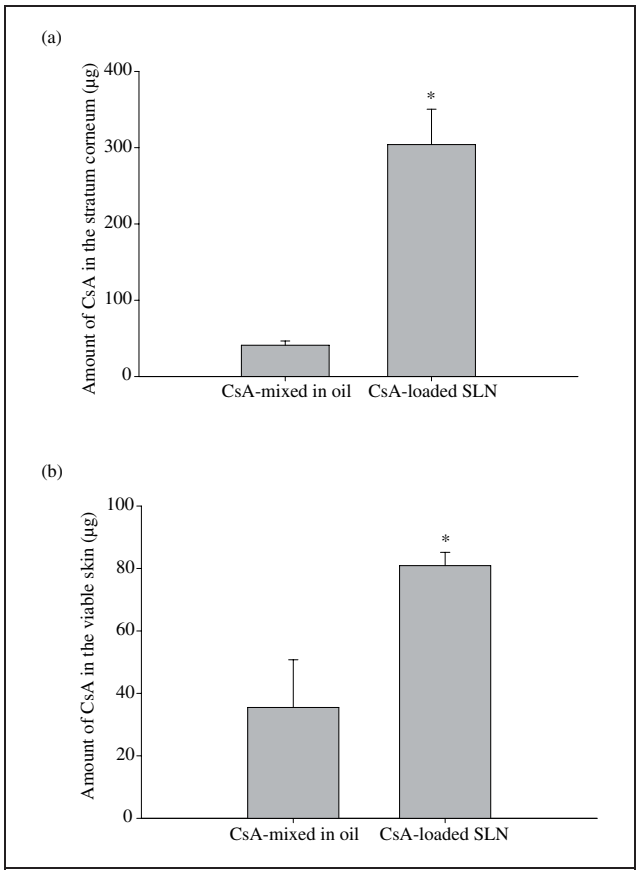

Fig. 3: Permeation study of CsA-loaded SLN and CsA-oil mixture. Both CsA-loaded SLN and CsA-oil mixture were applied to murine skin in the Franz-type diffusion cell. The two formulations were compared in the SC (a) and the viable skin (b). Data represents mean ± S.D. (n = 3), ($P < 0.001$)

(14%) after storage for 30 days. In contrast, the particle size of CsA-loaded SLN increased by 11% after 24 h incubation in 10% serum media (Fig. 1b).

The production of cytokines such as IL-4 and -5 by Th2 cells is closely related to immune disorders such as AD. As mentioned, Th2 producing cytokines are over-expressed in the patient with AD (Kimura et al. 2005). To investigate the possible cytotoxicity of CsA-loaded SLN (Fig. 2) D10.G4.1 murine Th2 cells were treated with CsA-loaded SLN. At 24 h postincubation, no significant changes in the cell viability was observed up to 50 μg/mL CsA as loaded forms in SLNs.

We employed Franz-type vertical diffusion model to assess the CsA permeation into the murine skin. After topical administration of CsA-loaded SLN or CsA-oil mixture in a Franz-type vertical diffusion cell, the amount of CsA penetrated into the SC and the viable skin were analyzed by HPLC (Fig. 3a and b). In murine skin, approximately 304 and 80 μg CsA were found in the SC and the viable skin when CsA-loaded SLN was applied to the Franz-type diffusion cells. However, only 41 and 35 μg CsA were found in the SC and in the viable skin when the CsA-oil mixture was applied to the cells. Therefore, CsA-loaded SLN permeation was about 7.4 times greater than that of the CsA-oil mixture in the SC and about 2.3 times greater than that in viable skin. Collectively, SLN formulation provided CsA greater penetration into both viable skin and SC compared with oil-mixture formulations. When viable skin and SC were compared, SLN formulation was more effective in providing CsA permeation into viable skin.

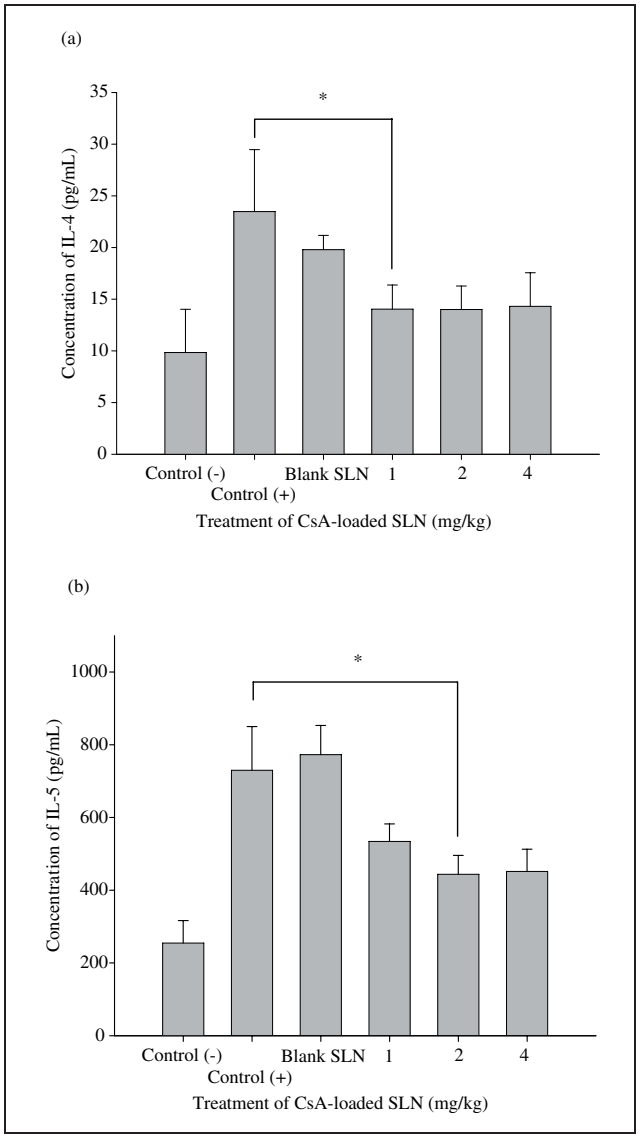

Fig. 4: *In vivo* immunological levels in sensitized mice after topical administration of CsA-loaded SLN. After sensitization, skin from each group was homogenized and centrifuged for analysis of cytokine levels. IL-4 (a) and IL-5 (b) in the supernatant were analyzed by ELISA. Results are presented as mean ± S.D. (n = 4)

After topical administration of CsA, Th2 cell cytokine levels were investigated in a murine skin model of AD. As shown in Fig. 4a, the IL-4 level was elevated two-fold (about 2.4) in the sensitized group (positive control) compared with the normal group (negative control). The IL-4 level in the group treated with empty SLN (without CsA) remained unchanged, whereas the IL-4 level significantly decreased in groups treated with CsA-loaded SLN ($P < 0.05$): When mice were administered with a topical dose of over 1 mg/kg CsA after sensitization, the IL-4 level decreased about 40% compared to the positive control group. The level of IL-5 in the sensitized group was also about 2.86 times higher than in the normal group (Fig. 4b). Furthermore, the IL-5 level also decreased when mice were administered a topical dose of over 2 mg/kg CsA after sensitization: The IL-5 concentration decreased about 30 and 39% in the groups treated with 1 and 2 mg/kg CsA. Thus, the maximal inhibitory effect on IL-5 was observed with topical administration of over 2 mg/kg CsA. Taken together, both IL-4 and IL-5 levels, which are overexpressed in the progression of AD, were also increased after sensitization, while topical administration of 2 mg/kg CsA-loaded SLN reduced these Th2 produced cytokine levels induced by sensitization.

3. Discussion

Topical administration through the skin enhances the therapeutic effect of bioactives while minimizing side effects from systemic administration, i.e. oral delivery (Lee et al. 2001). Dermatological therapy via topical route has therefore been widely used in the treatment of skin diseases (Magnusson et al. 2004). However, the stratum corneum (SC), the outermost layer of the skin, plays a protective role as a skin barrier against external solutes, including drugs, because of its very densely cross-linked protein structure (Brouwast et al. 2002). To increase penetration of drugs through this barrier, we developed a solid lipid nanoparticle system as a formulation for CsA, a relatively large molecule, and applied this system to topical administration.

CsA-loaded SLN exhibited physical stability adequate as a stable topical formulation (Table). Nano-sized CsA-loaded SLN (below 100 nm) were obtained, and the mean size was similar to that of the commercial ethanol-lipid mixture, NAT 8539, described in a previous report (Verma et al. 2004). Also, both empty- and CsA-loaded SLN exhibited a negative zeta potential value that was not significantly affected by the incorporation of CsA. According to previous studies on the effect of charge on permeation, negatively charged nanoparticles are more effective in permeating the skin (Kohli et al. 2004). In other studies, negatively charged chitosan nanoparticles had high effectiveness in vaccine delivery when administered on the skin (Cui et al. 2001, 2002). Thus, these studies showed that a slightly negatively-charged nanoparticle was effective in topical delivery, and our results preliminarily support the

ability of CsA-loaded SLN to permeate the skin. As shown in Fig. 3, topically administered CsA-loaded SLN more efficiently penetrated into both the SC and viable skin region than CsA-oil mixture. Our data is supported by a previous study comparing conventional oil-in-water cream and SLN, in which SLN greatly improved the penetration of a lipophilic compound in pig skin (Borgia et al. 2005).

Clinical trials of immunosuppressants have recently been performed for the treatment of severe AD. For example, tacrolimus was widely studied in an ointment formulation and effectively reduced chronic inflammatory disease symptoms (Champman et al. 2005). Based on its reduction of Th2 cell-related immune levels, CsA was selected as a calcineurin inhibitor for topical administration in this study. However, topical usage of CsA presented difficulties because CsA is a larger molecule than tacrolimus (about 804 Da). Therefore, SLN was chosen as a colloidal nano-carrier, and we expected that CsA-loaded SLN would penetrate the skin *in vivo* since SLN exhibited effective penetration ability in previous permeation studies.

To evaluate the immunosuppressing effect of CsA penetrated into the skin, Th2 cell-related IL-4 and -5 were analyzed as these cytokines play important roles in allergic inflammation and recruitment of eosinophils, and are thus elevated after allergic sensitization (Park et al. 2005; Arikan et al. 2003). Furthermore, IL-4 is involved in B-cell class switching and IgE production, whereas IL-5 increases eosinophils in AD. *In vivo* studies revealed that treatment with 1 mg/kg CsA decreased IL-4 to lower levels than in the sensitized mice group (positive control) ($P < 0.05$). Doses of over 2 mg/kg CsA also reduced the IL-5 level to less than that in sensitized mice (positive control) ($P < 0.01$). Based on the cytokine levels, SLN appears to transport CsA across the skin barrier where it inhibits immune responses related to AD.

Taken together, the present study suggests that SLN is an effective strategy for CsA penetration in topical administration of dermatological interest. In addition, CsA-loaded SLN may be effective for allergic inflammatory disorders such as AD.

4. Experimental

4.1. Materials

1,2,3-Tridecanoylglycerol (ticaprin), L-α-phosphatidylcholine (Egg-PC), *t*-butyl alcohol and Tween 80 were purchased from Sigma (St. Louis, MO, USA). Distearoyl-phosphatidylethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG) was purchased from Avanti Polar Lipid (Alabaster, AL, USA). Cyclosporin A was prepared by Hanmi Pharmaceutical Co., Ltd.

4.2. Preparation of CsA-loaded SLN

CsA-loaded SLNs were prepared by a melt homogenization procedure (Karande et al. 2004). Two hundred mg of tricaprin, 48 mg of Egg-PC, 46 mg of Tween 80, 6 mg of DSPE-PEG and 10 mg of CsA were dissolved in 1 mL of *t*-butyl alcohol. The mixture was incubated at −20 °C for 30 min, stored for 2 h in liquid N_2 and then lyophilized for 12 h. This mixture was vortexed after addition of 2 mL of distilled water and sonicated in a bath-type sonicator (Barinsin 3510, USA) at 50 °C for 1 h. These crude emulsions were homogenized for five cycles at 60–70 °C using a high-pressure homogenizer (Emulsiflex EFB3, Avestin, Canada). After homogenization, extruded dispersions were cooled in liquid N_2 for 2 h and then stored at 4 °C.

4.3. Analysis of size and zeta potential of SLN and CsA-loaded SLN

The size distribution of CsA-loaded SLN was measured by photon correlation spectroscopy using a submicron particle sizer (Nicomp 370, Particle Sizing System, USA). All results were recorded as volume distributions by volume-weighted Gaussian analysis. Zeta potential was measured with an electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at room temperature.

4.4. Entrapment efficiency of CsA-loaded SLN

CsA-loaded SLN were placed in centrifugal filter devices with a 30,000-MW cut-off (Microcon®, Bedford, MA, USA). Each tube was centrifuged at 10,000 rpm and the solution that passed through the insert was used for quantitative analysis. CsA was analyzed by an HPLC system equipped with a pump (LC-10AS, Shimadzu, Japan), autosampler (WatersTM 717 plus Autosampler, Waters, USA), column oven (655A-52 Column oven, Hitachi, Japan) and UV detector (SPD-10A, Shimadzu, Japan). A C_{18} reversed-phase column was used for separation (250 × 4.60 mm, 5 μm). Each sample was separated by isocratic elution with acetonitrile-water, 80 : 20 (v/v); the mobile phase flow rate was 1 mL/min at 60 °C. The eluate was monitored at 210 nm with a UV detector.

4.5. Stability study of CsA-loaded SLN

For stability evaluations, SLN and CsA-loaded SLN were stored at 4 °C for 1 month in the cold chamber or for 1 day in serum media. The mean size of SLN and CsA-loaded SLN was measured by photon correlation spectroscopy using a submicron particle sizer (Nicomp 370, Particle Sizing System, USA).

4.6. Cell culture and cytotoxicity

The D10.G4.1 cell line was obtained from Dr. E. J. Park (Institute of Molecular Biology and Genetics, Seoul, South Korea). Cells were maintained in RPMI medium supplemented with 0.05 mM 2-mercaptoethanol, 10 pg/mL IL-1α, 10% fetal bovine serum (FBS), and 10% rat T-STIM™ supplement from Beckton Dickinson (Bedford, MA, USA). The cytotoxicity of CsA-loaded SLN was determined using XTT tetrazolium salt-based assay as previously described (Seong et al. 2006). D10.G4.1 cells were plated onto 96-well plates at 1×10^4 cells/100 μL. After seeding, CsA-loaded SLNs were incubated with the cells for 12 h. Then, 50 μl of the XTT reaction mixture, prepared with sodium 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) and activation reagents that contained PMS (N-methyl dibenzopyranzine methyl sulfate), was added to each well. The excitation and emission were then measured at 690 and 450 nm, respectively.

4.7. Permeation study

Mouse whole skin was obtained from a female hairless mouse, aged 6 weeks. After cervical dislocation, the abdominal skin was surgically separated from the dorsal skin. The fat and subcutaneous tissues were then removed. Permeation studies were performed with a Franz-type vertical diffusion assembly. Each skin sample with a diffusion area of 3.14 cm^2 was placed in a thermostatted water bath at constant physiological temperature. The receptor medium was 25 mL of phosphate-buffered saline (pH 5.0) containing 0.1% sodium azide as a preservative and was stirred with magnetic bar. On the top of the assembly, 300 μL samples of soybean oil containing CsA or CsA-loaded SLN were added. After 8 h, each skin sample was separated from the Franz-type vertical diffusion assembly and carefully wiped to remove any excess formulation. The stratum corneum (SC) was tape-stripped with Scotch® crystal clear tape (19 mm diameter, 3M, Minnesota, USA) to separate the SC from the viable skin containing epidermis and dermis. All tape strips were extracted in 3 mL of 80% MeOH for analysis of CsA. The viable skin without SC was then homogenized and sonicated for 30 min. CsA in viable skin was analyzed by HPLC after filtration (cellulose acetate filter, 0.2 μm).

4.8. Sensitization of mice and application of CsA-loaded SLN

Mice were sensitized by epicutaneous administration of ovalbumin (OVA). Sensitization of female mice was performed as follows (Spergel et al. 1998): Briefly, after shaving the back skin of mice, 100 mg of OVA in 100 μL of normal PBS or 100 μL of PBS alone (control) was added to a patch of sterilized gauze (1 × 1 cm^2), applied to the shaven skin and fixed and secured with an elastic bandage to ensure skin contact and prevent oral ingestion. Each mouse had a total of three one-week exposures at two-week intervals.

After three epicutaneous sensitizations, the hair on the dorsal skin of the mice was shaved 24 h before topical application of CsA-loaded SLN. After OVA sensitization, SLNs were topically administered to the sensitized dorsal skin: negative control, positive control, blank SLN, CsA-loaded SLNs containing 1, 2, and 4 mg/kg doses of CsA. After scarification, collected murine dorsal skins were homogenized and centrifuged at 10,000 rpm. The supernatant from each homogenized skin was quantitatively analyzed by cytokine ELISA.

4.9. Statistics

Statistical analyses of data were performed using Student's t-test and ANOVA. A p value of less than 0.05 was termed significant.

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