Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: http://ees.elsevier.com/ajps/default.asp

Original Research Paper

Development of tacrolimus-loaded transfersomes for deeper skin penetration enhancement and therapeutic effect improvement *in vivo*

Wei Lei, Chuqin Yu, Huaqing Lin, Xiaoyuan Zhou*

Department of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 2 May 2013
Received in revised form
23 May 2013
Accepted 8 July 2013

Keywords:
Transfersomes
Tacrolimus
Transdermal
Deep skin layer target
In vivo therapy

ABSTRACT

The aims of this study were to prepare novel transfersomes (TFs) for tacrolimus to treat atopic dermatitis, and to observe the therapeutic effects on mice atopic dermatitis, as compared to commercial tacrolimus ointment (Protopic®) and liposomes-gel. Different kinds of surfactants—sodium cholate, Tween 80 and Span 80 were investigated to prepare TFs respectively. TFs-Tween 80 was selected as the optimal carrier owing to the best deformability and the highest drug retentions. Entrapment efficiency and diameter were also evaluated. The optimized TFs were further made into gel and *in vitro* drug release of TFs-gel after 24 h was higher than the commercial ointment. Cumulative drug release from TFs-gel after 12 h *in vitro* was 37.6%. The optimized TFs-gel illustrated remarkably highest drug skin retentions when compared with liposomes-gel and commercial ointment *in vivo* skin retention experiments. The amounts of tacrolimus in epidermis and dermis from TFs-gel were 3.8 times and 4.2 times respectively as much as ointment, while liposomes-gel was only 1.7 times and 1.4 times respectively as compared to ointment. Topical application of TFs-gel displayed the best therapeutic effect on mice atopic dermatitis induced by repeated topical application of 2,4-dinitrofluorobenzene. Thus TFs displayed superior performance and effective skin target for topical delivery of tacrolimus.

1. Introduction

Atopic dermatitis (AD) was a chronic and relapsing skin disease with severe itching and eczematous lesions [1]. The increasing serum IgE level and marked perivascular T-cell infiltration were the characteristics in pathological changes skin of AD [2]. Situations, such as somnipathy caused by itch scratch significantly influenced life qualities in both adult and

* *Corresponding author*. Provincial Key Laboratory of Advanced Drug Delivery, Department of Pharmacy, Guangdong Pharmaceutical University, No. 180, Outside Loop East Road, Higher Education Mega Center, Guangzhou 510006, China. Tel.: +86 20 39352518.
E-mail address: huaqing_@163.com (H. Lin).
Peer review under responsibility of Shenyang Pharmaceutical University

Production and hosting by Elsevier

1818-0876/$ – see front matter © 2013 Shenyang Pharmaceutical University. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ajps.2013.09.005

infant patients with AD [3]. Furthermore, induced scratch damaged the skin barrier and exacerbated the dermatitis [4]. Tacrolimus, also known as FK506, was a powerful macrolide lactone immunosuppressant isolated from *Streptomyces tsukubaensis* and showed notably therapeutic efficacy for immune-inflammatory conditions including AD [5,6]. Tacrolimus functioned primarily on T-cells in affected skin, restraining T-cells activation and controlling the release of pro-inflammatory cytokines. Moreover, tacrolimus could influence other immune cells pathways that were related to the pathogenesis of AD, such as blocking cytokine generation and release of basophils, eosinophils and mast cells, decreasing the number of inflammatory dendritic epidermal cells in AD injured layers [7].

At present, there was only one tacrolimus commercial preparation for topical application, tacrolimus ointment. In 1999, tacrolimus ointment had been approved by the Food and Drug Administration to treat moderate to severe cases of AD. Current studies had also suggested that topical application of ointment might be effective in the treatment of acute contact dermatitis, erosive lichen planus, isolated ichthyosis linearis circumflexa, and Lazarous and Kerdel [8]. However, physicochemical properties of tacrolimus, high molecular weight (822.95 g/mol) and strong lipophilicity (partition coefficient log$P$ = 3.96 ± 0.83), made it difficult to overcome the skin barrier and penetrate into deeper skin layers [9]. What's more, the skin barrier would extend at diseased regions [10]. It was highly challenging to overcome the skin barrier-stratum corneum (SC) and reach the dermis in sufficient active drug concentrations. A novel delivery system which could help more tacrolimus penetrate across SC and target site of action was necessary to provide.

Transfersomes (elastic or ultraflexible liposomes) were a new class of lipid vesicles introduced by Gregor et al. in 1992 [11]. TFs might offer advanced local therapies with agents that were unable to efficiently penetrate the SC via passive diffusion. Ketoprofen in TFs formulation had gained marketing approval by the Swiss Regulatory Agency [12]. TFs consisted of lipids and a membrane softening agents, where lipids were the major components of vesicle membrane and softening agents functioned as a destabilizing factor to increase the deformability of TFs membrane. The combination of appropriate lipids with membrane softening agents could enable TFs to penetrate pores that were five times smaller than their own diameters. Moreover, the diameters maintained even after TFs passed through small pores against fragmentation [13,14]. Surfactants were suggested as examples of softening agents, such as sodium cholate (SDC), Span 80 and Tween 80 [15]. When TFs dispersions were applied to skin non-occlusively, the excess of applied water started to evaporate immediately and osmotic gap was built up to facilitate TFs to across the skin SC primary barrier [16]. The high deformability and hydration driving force of TFs both allowed and prompted the drug-loaded vesicles to across the skin barrier [17].

The present study was to screen a suitable surfactant for TFs to enhance skin delivery of tacrolimus. Three kinds of surfactants were investigated in the preparation of TFs. The entrapment efficiency, particle size, deformability index and drug retentions of different TFs were compared. The optimal TFs were prepared to gel and drug release *in vitro* were compared with liposomes-gel and ointment. Then the amounts of tacrolimus depositing in different skin layers *in vivo* percutaneous permeation and therapeutic effects on mice AD models were used to evaluate the efficiency and superiority of TFs-gel.

2. Materials and methods

2.1. Materials and animals

Tacrolimus was purchased from Huadong pharmaceutical Co., Ltd. (Hangzhou, China). Protopic® (0.1% tacrolimus ointment) was produced by Astellas Toyama Co., Ltd. (Toyama city, Japan). Lipoid E80 containing more than 80% phosphatidylcholine (PC) was bought from Shanghai Toshisun Biology & Technology Co., Ltd. (Shanghai, China). 2,4-Dinitrofluorobenzene (DNFB) was purchased from Xiya Chemical Industry co., Ltd. (Chengdu, China). HPLC reagents were supplied by Tedia Company Inc (Ohio, America). All other chemicals and solvents were of analytical grade or better.

SD rats (male, 350–400 g) and KM mice (male, 20–23 g) were obtained from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China) and housed under specific pathogen-free conditions. The rats were allowed to acclimatize for at least 3 days prior to any study procedures. The study protocol was approved by the Animal Ethics Committee of Guangdong Pharmaceutical University.

2.2. Formulation of tacrolimus-loaded TFs

Tacrolimus-loaded TFs were prepared by thin film dispersion–hydration method. Briefly, drug, lipoid E80, surfactants and vitamin E were completely dissolved in round bottom flask with 20 ml of dehydrated alcohol. The alcohol

Table 1 – Different kinds of surfactants and their influences on characteristics of vesicles (n = 3).

Formulation name[a]	EE%	Particle size/nm	Deformability index	Drug retentions (μg/cm^2)
TFs-SDC	70.60 ± 1.10	135.6 ± 2.9	6.25 ± 0.09	5.11 ± 0.38
TFs-Tween 80	78.86 ± 1.62	123.1 ± 2.2	9.22 ± 0.17	6.66 ± 0.68
TFs-Span 80	83.88 ± 2.36	260.6 ± 4.0	7.93 ± 0.14	5.87 ± 0.51
Liposomes	71.76 ± 1.29	159.7 ± 3.5	4.30 ± 0.06	4.22 ± 0.32

[a] In addition to the different kinds of surfactants, the other ingredients of formulations were the same (lipoid E80: 1.200 g, surfactant: 0.372 g, FK506: 72 mg, vitamin E: 20 mg, PBS(pH6.5): 20 ml).

was evaporated using rotatory evaporator and homogeneous thin lipid film was obtained. The film was hydrated with 20 ml of pH 6.5 phosphate buffer for 1 h at the temperature of 20 °C to get rude dispersions. The dispersions was sonicated using a Scientz-IID sonicator (Scientz Biotechnology Co., LTD, Ningbo, China) for 9 min under 400 w. Extrusion through 0.45 μm pore size Nylon-66 membranes was followed for size reduction.

Three kinds of surfactants were inspected, that were SDC, Tween 80 and Span 80. Liposomes loaded tacrolimus were also formulated and the surfactant was substituted with cholesterol. The resulting TFs dispersions and liposomes dispersions were in light blue opalescence, except TFs-SDC dispersions were in brown yellow which was the color of SDC itself. Differences of TFs were listed in Table 1.

2.3. Determination of drug encapsulation efficiency

The encapsulation efficiency (EE%) of TFs and liposomes was determined using the dialysis technique for separating the non-entrapped tacrolimus. 1 ml of tacrolimus-loaded TFs or liposomes dispersions were dropped into a dialysis bag (molecular weight cut off: 10 kDa) immersed in 10 ml of 25% v/v ethanol solution and shook at 50 rpm [18]. 0.5 ml of ethanol solution was taken out after 8 h and analyzed by HPLC to account the mass of unentrapment drug (W_f). Then 1 ml of tacrolimus-loaded TFs or liposomes dispersions were demulsified with 25 ml methanol and the obtained solution was detected as total drug in dispersion (W_t). The encapsulation efficiency (EE%) could be counted by the following equations:

$$EE\% = \frac{W_t - W_f}{W_t} \times 100\%$$

2.4. Characterization of tacrolimus-loaded TFs

2.4.1. Particle size distribution

Particle size distribution of TFs and liposomes were measured using dynamic light scattering (Beckman Coulter Delsa™ Nano C, USA). Samples were diluted by 1-fold with distilled water before measurement. All of samples were measured 3 times at 25 °C under a fixed scattering angle of 90°.

2.4.2. Relative deformability

The relative deformability of TFs was compared with liposomes by extrusion measurement [19]. Flexibility was observed by extrusion of vesicles through 0.22 μm Nylon-66 syringe filters (Filtration area: 1.3 cm^2, Jinteng Experiment Equipment Co., Ltd, Tianjin, China) at a constant pressure of 0.2 MPa. The extrusion volume in 5 min was recorded for TFs dispersions, liposomes dispersions, respectively. Deformability index (DI) was counted using the following formula:

$$DI = J * \frac{r_v}{r_p}$$

where, J = the volume of the dispersions extruded during 5 min; r_v = the size of the vesicle; r_p = pore size of the filter.

2.4.3. In vitro *percutaneous permeation*

Skin samples obtained from the abdominal region of male SD rats were used to evaluate the *in vitro* permeation of tacrolimus delivered via TFs, liposomes dispersions. The permeation studies were conducted using the Franz diffusion cells (Xiekai Scientific, China). The skin was mounted on diffusion cells with the dermis facing the receptor compartment and the surface area of skin was 3.14 cm^2. The same quality of dispersions was placed. Receptor compartment was full of 25% alcohol solution. After 24 h, the permeation experiment was end and receptor medium was withdrawn waiting for detection. Skin samples were removed and immersed in methyl alcohol waiting for detection.

2.4.4. *Microscopic morphology*

Transmission electron microscopy (TEM) images were taken employing a JEM-1400 microscope (JEOL, Japan) operating at an accelerating voltage of 120 kV. The sample was prepared according to Laouini's work [20]. The optimized TFs dispersions were firstly diluted 10 times with distilled water and dropped onto a carbon-coated copper grid. The excess dispersion was removed with a filter paper after 1-min. Negative staining using a 2% phosphotungstic acid solution (w/w) was directly made on the deposit for 45 s. Then the air-dried samples were directly examined under the TEM.

2.5. Formulation of vesicular gel

2.5.1. *Preparation of vesicular gel*

In order to make dispersion suitable for topical application, TFs and liposomes dispersions were made into gel. Carbopol®940 was added into the distilled water and was stirred over night with magnetic stirrer. TFs and liposomes dispersions were added to carbopol mixture introduced above. The concentration of carbopol®940 was 1% (wt.). Triethanolamine was put in to neutralize pH value of 6.0. Then vesicular gel was obtained.

2.5.2. In vitro *release of vesicular gel*

In vitro release of vesicular gel was measured utilizing vertical glass Franz diffusion cells introduced above. Nylon66 filters (0.20 μm pore diameter, Millipore Isopore™, USA) were bathed in isopropyl myristate to imitate the lipophilic property of SC [21]. Weighted quantity (0.3 g) of vesicular gel and tacrolimus ointment were applied to the donor compartment while receptor compartment was full of 25% alcohol solution. Receptor compartment was maintained at 32 ± 0.5 °C and stirred at 200 rpm constantly. Receptor solution was taken at predetermined time intervals.

2.6. In vivo *percutaneous permeation*

An area of 2 × 3 cm in the dorsal skin of SD rat was hair removed. The same quality of vesicular gel and tacrolimus ointment were applied evenly. After 24 h, the permeation experiment was end and the rats were anesthetic by inhalation of chloroform. 1 ml of blood and skin over the site of use were collected from each rat. The skin layers were separated referring to the work of Babu et al. [22]. The SC was

removed by tape stripping and 10 strips were collected. The remained skin was frozen at −50 °C and separated into epidermis and dermis horizontally using a cryo-microtome (CM1900 Leica, Germany). The epidermis layers were removed by 4 sections 25 μm thick each. The portions left were collected as dermis layers. Three parts of skin layers were chopped and extracted in methanol with homogenization (IKA® Wiggens D-120, Germany). The samples were kept refrigerated for 12 h and then centrifuged to get supernatant for analysis of tacrolimus content. Blood sample process was referred to Lee et al. [23].

2.7. Pharmacodynamic study

Repeated topical applications of DNFB were used to induce AD with appropriate changes from the method presented by Pokharel et al. [24]. Firstly, sensitization was caused by applying 25 μl of 0.5%DNFB dissolved in acetone/olive oil (4:1) to the hair-removed abdomen of mice once per day on a continuous three-day. Then mice were bred for 3 days normally. At the sixth day, the backs of mice were unhaired with an area of 2 × 2 cm. 40 μl of 0.25% DNFB were placed uniformly once per 3 days to stimulated AD. At the same time, 5 μl of 0.25% DNFB were applied to the inside of the right earlobe of mice while the same quantity of acetone/olive oil (4:1) was applied to the left side. Four times later, AD model was prepared. It should be noted that blank control group was given acetone/olive oil.

FK506 loaded TFs-gel group, liposomes-gel group and Protopic® group were spread with corresponding preparation twice one day while the model group and blank control group didn't get any treatments. Administration dose was 0.25 g/back and 0.05 g/earlobe.

After the final administration, morphological changes of mice back skin, left and right ears were observed. The mice were then sacrificed and ears of both sides were cut off using a hole punching device (diameter as 6 mm). Mass and thickness differences between left and right ear were used to evaluate the induced ear swelling. Skin tissues from the backs of mice were excised and subjected to histological examination.

2.8. Analytics

Tacrolimus was quantified using a Dionex Ultimate3000 HPLC system equipped with a LPG-3400SD quaternary pump and a DAD-3000 diode array detector. The assay was carried out at a wavelength of 210 nm, using a Agilent SB C_8 column (4.6 × 250 mm, 5 μm) maintained at a temperature of 60 °C, with a mobile phase composed of acetonitrile/water (60:40, v/v), a flow rate of 1 ml/min and injection volume of 20 μl.

2.9. Statistics

Statistical significance was determined by one-way ANOVA followed by Student's *t*-test as post hoc analysis. Differences were considered as significant at $p < 0.05$.

3. Results and discussion

3.1. Characterization of TFs

TFs were prepared by using three different kinds of surfactants, namely SDC, Tween 80 and Span 80. Table 1 showed the effects of different surfactants types on characterizations of TFs.

3.1.1. Drug encapsulation efficiency

TFs consisting different kinds of surfactants displayed diverse EE%. Maximum EE% (83.88%) was obtained from TFs-Span 80, followed by TFs-Tween 80 (78.86%) and TFs-SDC (70.60%). The phenomenon was similar to El Zaafarancy et al. [25].

The results could be explicated as that the entrapment of lipophilic drug into lipid vesicles was facilitated by drug distribution coefficient between lipid phase and aqueous solution [26]. The HLB of Span 80, Tween 80 and SDC was 4.3, 15.0 and 16.7. Correspondingly, the affinity of surfactants with lipids decreased in sequence. Based on the affinity of surfactants with lipids and the strong lipophilic property of drug (log*P* = 3.96 ± 0.83), tacrolimus would be more prevalent dispersing in double-layer lipids established by Span 80 and PC. SDC had a steroidal structure similar to the macrolide tacrolimus and competed for the position in the bi-layered vesicles. This displacement between species led to low EE% to some extent [27].

3.1.2. Particle size

There were significant differences in particle size between TFs consisting different surfactants. TFs containing SDC, Tween 80 and Span 80 expressed a mean particle size of 135.6, 123.1 and 260.6 nm, and the mean particle size of liposomes was 159.7 nm.

In general, particle size increased along with surfactants possessing lower HLB. The trend between HLB and particle size could be attributed to the increase in surface free energy accompanying increasing hydrophobicity of surfactant. Surfactants interacted with lipid head groups in the membrane would increase packing density of layer, which could lead to high surface free energy [28]. Thus, the increasing surface free energy led to fusion between lipid bilayers and larger size [29]. While Span 80 had the strongest hydrophobicity, the size expansion of TFs-Span 80 was obviously compared with other TFs.

3.1.3. Relative deformability

Deformability was one of the most important characteristics for TFs to differ from other vesicles like liposomes. When surrounding stress was enforced on vesicles to penetrate the skin pores, TFs could spontaneously undergo deformation to avoid the risk of structure rupture. Table 1 showed the relative deformability of TFs affected by different types of surfactants.

DI% had a positive correlation with deformability. The deformability of TFs was the 1.4–2.1 times that of the liposomes. As described by Benson [30], the deformability of TFs could attribute to the membrane fluidity. Membrane of TFs was constituted by two amphiphilic components (PC plus

surfactant) which owned sufficiently different packing characteristics and could transform bilayer structure into a single layer. TFs-Tween 80 owned the highest deformability (DI: 9.22) because Tween 80 had nonbulky hydrocarbon chains and no steroid-like structure like SDC (DI: 6.25) that were unbeneficial to membrane fluidity. TFs-Span 80 (DI: 7.93) showed the worst deformability which could be a result of its high hydrophobicity. Its high hydrophobicity could reduce the formation of transient hydrophilic holes which was responsible for membrane fluidity [28].

3.1.4. *Quantification of drug retained in skin* in vitro

The effects of TFs containing different surfactants on permeation *in vitro* were investigated. The amounts of tacrolimus retained in skin for different vesicles were shown in Table 1. TFs significantly enhanced the accumulation of drug into the skin layers. Tacrolimus retention levels in skin of TFs demonstrated 1.2–1.6 times higher than liposomes. Unlike liposomes, TFs not only acted as drug vectors, but also acted as penetration enhancers. Penetration enhancement could be ascribed to the presence of surfactant contributing good deformation to TFs [31, 32]. TFs dispersions were partly dehydrated by water evaporation after placed on the skin surface, and transcutaneous hydration gradient was produced in skin [33]. The hydration gradient drove vesicles to carry drug to enter the deeper skin avoiding dehydration and fusion [16]. TFs owning better membrane deformability could penetrate the skin through the hydrophilic pathways or pore between the skin cells without losing its vesicle integrity. While liposomes with less flexible bilayers would fracture during transport through skin pore [34].

There existed apparently different drug retentions among above TFs and the order was as follows: TFs-Tween 80 (6.66 ± 0.68 μg/cm^2) >TFs-Span 80 (5.87 ± 0.51 μg/cm^2) >TFs-SDC (5.11 ± 0.38 μg/cm^2) >Liposomes (4.22 ± 0.32 μg/cm^2). These significant variations were affected synthetically by EE %, particle size and deformability. TFs-Tween 80 owned the highest drug retentions. This could be explained by its small particle size and good shape deformability which were beneficial to delivering more tacrolimus through skin barrier and depositing in skin. Our results were inconsistent with the report by Jain et al. (Span 80 > SDC > Tween 80) [15]. This observation probably argued on the differences in drug nature between dexamethasone and tacrolimus.

However, the receptor fluid collected for TFs and common liposomes did not detect the presence of any drug. This might be explained that the amount of tacrolimus penetrating through skin and entering to receptor was tiny or none. These results exactly corresponded with local use of tacrolimus treating skin disease and didn't cause system adsorption [35].

3.2. *Morphology observation*

When all of these characterizations (EE%, particle size, deformability index and drug retentions) were taken into accounts together and more considerations were given to drug retentions. TFs-Tween 80 was chosen as the optimal carrier and used for further research. TEM for the TFs-Tween 80 dispersions exhibited typical single chamber structure (Fig. 1).

3.3. In vitro *drug release*

TFs-Tween 80 and liposomes dispersions were prepared to gel containing Carbopol® 940 (1% wt). Both TFs-gel and liposomes-gel displayed well adhesive capacity while spreading over skin.

In vitro release studies of TFs-gel, liposomes-gel and marketed ointment were investigated (Fig. 2). The drug release from TFs-gel and liposomes-gel was 2.8 times and 2.3 times as much as ointment ($p < 0.05$).

The release profiles of tacrolimus from TFs-gel and liposomes-gel showed biphasic release processes [20], where initial burst release of the surface-adsorbed drug was observed, followed by slow diffusion from the lipid vesicles. At the initial 4 h, the little higher drug release of liposomes-gel was observed. This could be attributed to more unentrapped drug distributing in the gel system as liposomes had a lower EE%. Unentrapped drug could pass through release interface earlier compared with drug entrapped in vesicles. Afterward, lipid vesicles diffusion in gels played an important role in the release profiles and drug release rate slowed down. The cumulative drug release of TFs-gel, liposome-gel and ointment were (116.72 ± 7.49) μg, (95.75 ± 5.89) μg and (41.34 ± 3.59) μg in 24 h. TFs with higher EE% and better deformability could carry more drugs through filters quickly as compared with liposomes.

3.4. *Quantification of drug in different skin layers*

In vivo skin retention experiments were conducted to further study the distribution of tacrolimus in different skin layers (SC, epidermal and dermal) after administration of TFs-gel, liposomes-gel and ointment (Fig. 3). TFs-gel and liposomes-gel obviously facilitated the penetration of tacrolimus into skin as compared to ointment ($p < 0.05$).

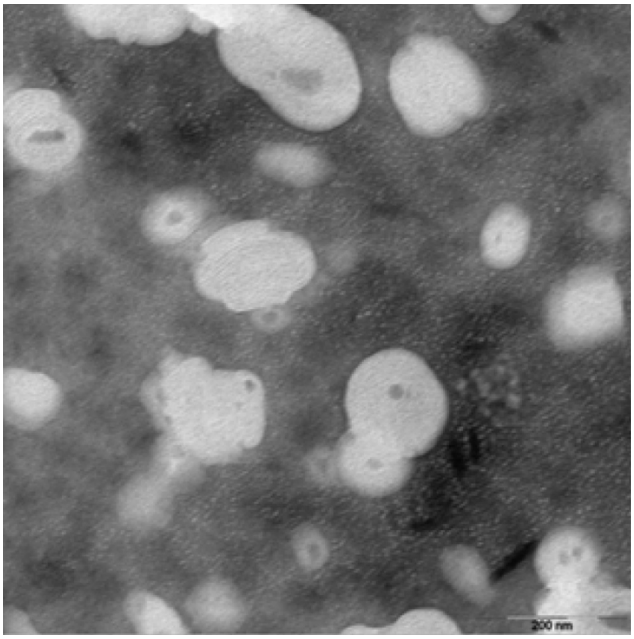

Fig. 1 – TEM morphology of TFs-Tween 80 (30,000×).

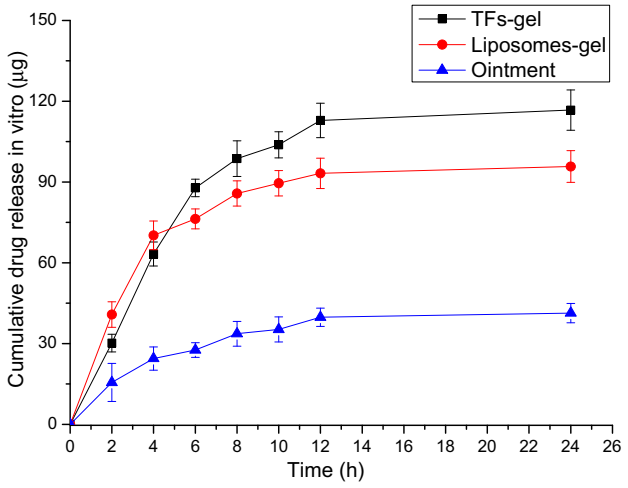

Fig. 2 – *In vitro* cumulative drug release ($n = 3$).

SC was the rate-limiting step in percutaneous delivery. In the SC, three final preparations displayed almost same distributions of tacrolimus. This phenomenon might indicate that reservoir capacity of the SC arrive saturation state. Tacrolimus mainly reserved in SC (as a drug deposit pool), on account of SC lipid-rich environment. Then slow permeation into the deeper epidermis and dermis would occur [36]. The obtained results of the three preparations revealed that the amounts of tacrolimus decreased gradually along with the deeper layers (SC > epidermis > dermis) which was in agreement with the findings of Betz et al. [37]. As diffusivity and concentration of drug in deeper skin were small, the carriers were hampered in further spontaneous motion. Choi reported TFs could carry drug to reach a depth of at least 30 μm [38]. In the epidermis and dermis, amounts of tacrolimus for TFs-gel were (1.73 ± 0.21) μg/cm^2 and (1.23 ± 0.13) μg/cm^2, which were 3.8 times and 4.2 times as ointment. While liposomes-gel were only 1.7 times and 1.4 times that of ointment ($p < 0.05$). Liposomes would rupture while squeezing the nano-pores

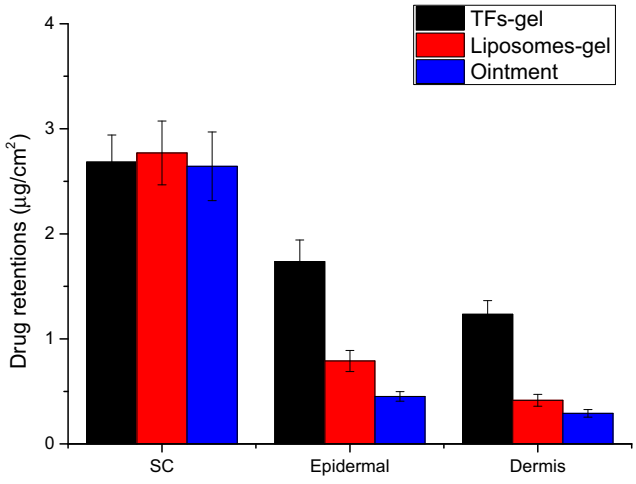

Fig. 3 – The amount of drug retention different skin strata *in vivo* skin retention experiments ($n = 6$).

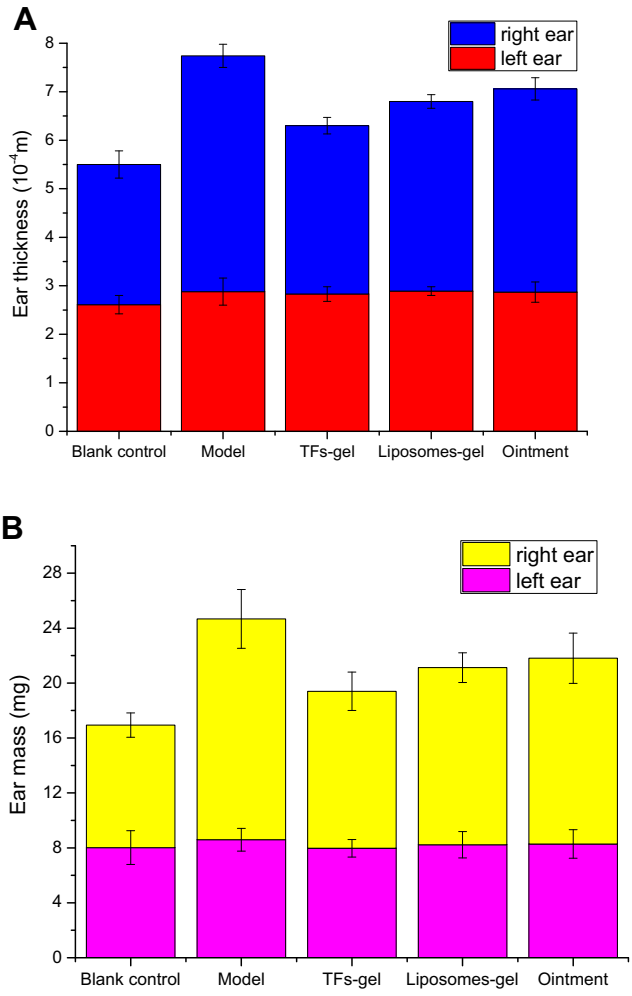

Fig. 4 – Ear swelling of mouse after last treatments with different tacrolimus preparations ($n = 10$).

which were much smaller than vesicles sizes due to their rigid structures. They carried fewer amounts of applied drug into the deeper layer. TFs could be released from gel system and overcome the nano-porous barrier to follow the natural water gradient across the epidermis by the greater flexibility and movement of the bilayer. Then more drugs would be transferred to deeper skin [39].

Like receptor fluid collected *in vitro* permeation studies, blood collected in three preparations groups did not detect the presence of any drug either.

3.5. In vivo *therapeutic efficacy*

To evaluate the therapeutic effects of TFs-gel loaded tacrolimus on atopic dermatitis mice model, we investigated ear swelling, morphological appearances and histopathological features of treated skin. Liposomes-gel and ointment were assessed in the same way.

Fig. 4 showed the ear swelling changes (including ear mass and thickness) in the whole experiments. The significantly increasing ear swelling after stimulation was one of

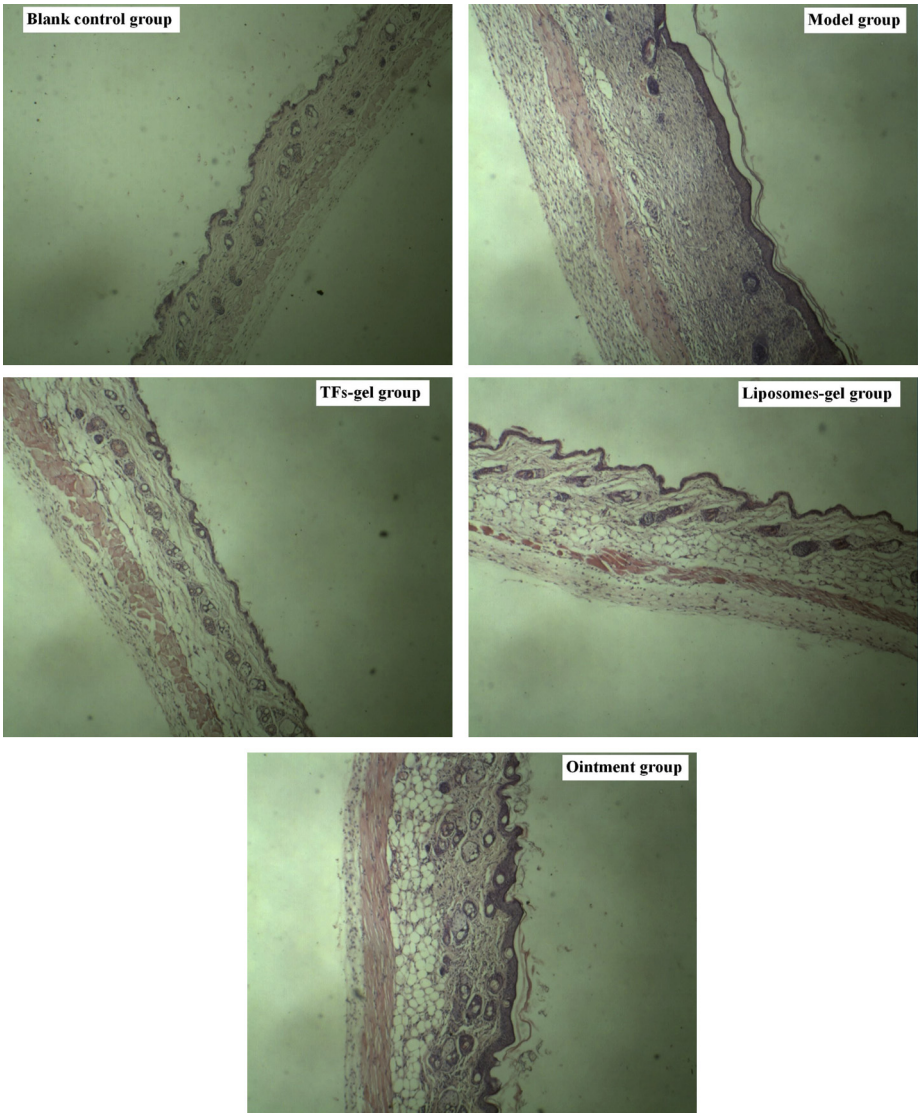

Fig. 5 – The mice skin histopathological images of different groups ($n = 10$).

characterizations of dermatitis model induced by DNFB [40]. After repeated administrations for seven days, TFs-gel displayed the best therapeutic effects by depressing ear swelling to the minimum, as compared with other preparations. Ear thickness decreased from 0.486 mm to 0.347 mm, and ear mass cut down from 16.08 mg to 11.43 mg. Liposomes-gel expressed a slightly better effect than ointment which was reported to be clinically effective and safe for atopic dermatitis [41].

Stimulation with DNFB for several times, dorsal dermal of model mice appeared erythema, edemaand scabs. Besides, scratching behaviors was obviously. Histopathological images (Fig. 5) showed significant thickening of the skin layers, as well as an accumulation of inflammatory cell nucleus in the inflamed skin layer. These pathological changes were in accordance with the previous literatures [42].

As was shown in Fig. 6, after administration with TFs-gel for 7 days, injured skin started to get better and enabled the wound to grow scab. Then scabs exuviated and exposed the healing skin. New hair began to grow at most of damaged skin surface. The histopathology of the skin showed the thickness of skin layer became significantly thinner and the amounts of inflammatory cell nucleus reduced. Part of the skin surface of liposomes-gel group grew new hair and skin layers were thicker than TFs-gel. Ointment group didn't yet grow new hair and inflammatory cell nucleus infiltration was still evident. Liposomes-gel and ointment took effect slowly as compared to TFs-gel.

These results verified the retentions of tacrolimus in different skin layers for three preparations. TFs-gel carried more tacrolimus into deeper layer and took effects quickly. Tacrolimus was an anti-T-cell drug and suppressed the release of inflammatory cytokine which was necessary for the immediate and chronic allergic reactions of inflammatory cells [43]. All tacrolimus-loaded preparations decreased thickening of the epidermis/dermis and suppressed the increase of inflammatory cells at affected sites.

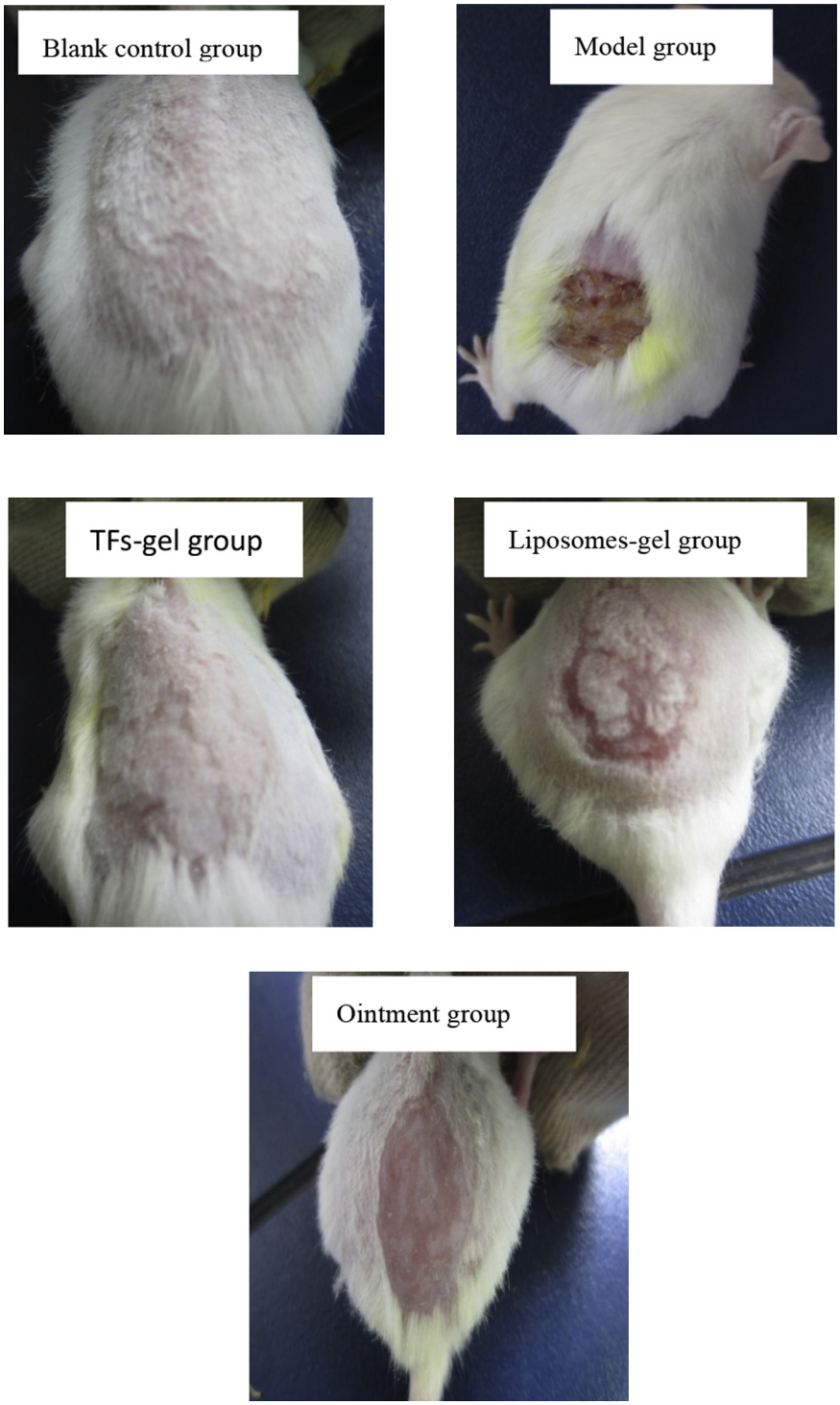

Fig. 6 – The mice dorsal skin morphological changes of different groups (n = 10).

4. Conclusion

In this work, TFs displayed special deformability responding to an external stress and worked as an enhancer of percutaneous penetration. Different types of surfactants for TFs could lead to distinct entrapment efficiency, diameter, deformability and drug retentions. Tween 80 was more effective for TFs to load tacrolimus as compared to other surfactants.

Tacrolimus could be entrapped into TFs with good deformability to penetrate skin pores much narrower than vesicles diameter. More drugs could be transported to deeper skin layers. TFs-gel loaded tacrolimus displayed more effective retentions in epidermis and dermis compared with traditional liposomes-gel and commercial ointment. *In vivo* therapy of mice dermatitis, TFs-gel took effect more quickly than liposomes-gel and commercial ointment. Ear swelling

and pathological skin were improved markedly. We could conclude that TFs might be a useful carrier for tacrolimus to treat for atopic dermatitis.

In further study, CLSM (confocal laser scan microscope) could be used to visualize the cutaneous uptake and confirm target to deeper skin layers. The drug retentions in pathological changes skin with AD also should be determined, as permeability barrier of diseased skin were different from the healthy.

REFERENCES

[1] Saeki H, Furue M, Furukawa F, et al. Guidelines for management of atopic dermatitis. J Dermatol 2009;36:563–577.

[2] Akdis CA, Akdis M, Simon D, et al. T cells and T cell-derived cytokines as pathogenic factors in the nonallergic form of atopic dermatitis. J Invest Dermatol 1999;113:628–634.

[3] Drake L, Prendergast M, Maher R, et al. The impact of tacrolimus ointment on health-related quality of life of adult and pediatric patients with atopic dermatitis. J Am Acad Dermatol 2001;44:S65–S72.

[4] Wahlgren CF. Itch and atopic dermatitis: an overview. J Dermatol 1999;26:770–779.

[5] Rubins A, Gutmane R, Valdmane N, et al. Pharmacokinetics of 0.1% tacrolimus ointment after first and repeated application to adults with moderate to severe atopic dermatitis. J Invest Dermatol 2005;125:68–71.

[6] Beck LA. The efficacy and safety of tacrolimus ointment: a clinical review. J Am Acad Dermatol 2005;53:S165–S170.

[7] Sengoku T, Kishi S, Sakuma S, et al. FK506 inhibition of histamine release and cytokine production by mast cells and basophils. Int J Immunopharmacol 2000;22:189–201.

[8] Lazarous MC, Kerdel FA. Topical tacrolimus Protopic. Drugs Today (Barc) 2002;38:7–15.

[9] Goebel AS, Neubert RH, Wohlrab J. Dermal targeting of tacrolimus using colloidal carrier systems. Int J Pharm 2011;404:159–168.

[10] Linde YW. Dry skin in atopic dermatitis. Acta Derm Venereol Suppl (Stockh) 1992;177:9–13.

[11] Cevc G, Blume G. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim Biophys Acta 1992;1104:226–232.

[12] Kneer W, Rother I, Rother M, et al. A multiple-dose, open-label, safety, compliance, and usage evaluation study of epicutaneously applied Diractin (ketoprofen in Transfersome) in joint/musculoskeletal pain or soft tissue inflammation. Curr Drug Saf 2009;4:5–10.

[13] Hofer C, Randenborgh H, Lehmer A, et al. Transcutaneous IL-2 uptake mediated by Transfersomes depends on concentration and fractionated application. Cytokine 2004;25:141–146.

[14] Rother M, Lavins BJ, Kneer W, et al. Efficacy and safety of epicutaneous ketoprofen in Transfersome (IDEA-033) versus oral celecoxib and placebo in osteoarthritis of the knee: multicentre randomised controlled trial. Ann Rheum Dis 2007;66:1178–1183.

[15] Jain S, Jain P, Umamaheshwari RB, et al. Transfersomes–a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation. Drug Dev Ind Pharm 2003;29:1013–1026.

[16] Cevc G, Gebauer D. Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. Biophys J 2003;84:1010–1024.

[17] Kawasaki H, Nagao K, Kubo A, et al. Altered stratum corneum barrier and enhanced percutaneous immune responses in filaggrin-null mice. J Allergy Clin Immunol 2012;129:1538–1546 e1536.

[18] Mura P, Maestrelli F, Gonzalez-Rodriguez ML, et al. Development, characterization and in vivo evaluation of benzocaine-loaded liposomes. Eur J Pharm Biopharm 2007;67:86–95.

[19] Modi CD, Bharadia PD. Transfersomes new dominants for transdermal drug delivery. Am J Pharm Tech Res 2012;2:71–91.

[20] Laouini A, Jaafar-Maalej C, Sfar S, et al. Liposome preparation using a hollow fiber membrane contactor – application to spironolactone encapsulation. Int J Pharm 2011;415:53–61.

[21] Kilfoyle BE, Sheihet L, Zhang Z, et al. Development of paclitaxel-TyroSpheres for topical skin treatment. J Control Release 2012;163:18–24.

[22] Babu RJ, Kikwai L, Jaiani LT, et al. Percutaneous absorption and anti-inflammatory effect of a substance P receptor antagonist: spantide II. Pharm Res 2004;21:108–113.

[23] Lee MJ, Straubinger RM, Jusko WJ. Physicochemical, pharmacokinetic and pharmacodynamic evaluation of liposomal tacrolimus (FK 506) in rats. Pharm Res 1995;12:1055–1059.

[24] Pokharel YR, Lim SC, Kim SC, et al. Sopungyangjae-tang inhibits development of dermatitis in Nc/Nga mice. Evid Based Complement Alternat Med 2008;5:173–180.

[25] Zaafarany GM, Awad GA, Holayel SM, et al. Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. Int J Pharm 2010;397:164–172.

[26] Scognamiglio I, De Stefano D, Campani V, et al. Nanocarriers for topical administration of resveratrol: a comparative study. Int J Pharm 2012;440:179–187.

[27] Subuddhi U, Mishra AK. Micellization of bile salts in aqueous medium: a fluorescence study. Colloids Surf B Biointerfaces 2007;57:102–107.

[28] Lichtenberg D, Opatowski E, Kozlov MM. Phase boundaries in mixtures of membrane-forming amphiphiles and micelle-forming amphiphiles. Biochim Biophys Acta 2000;1508:1–19.

[29] Yoshioka T, Sternberg BF, Lorence AT. Preparation and properties of vesicles (niosomes) of sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85). Int J Pharm 1994;105:1–6.

[30] Benson HA. Transfersomes for transdermal drug delivery. Expert Opin Drug Deliv 2006;3:727–737.

[31] Maswadeh H, Demetzos C, Dimas K, et al. Accumulation of vinblastine into transfersomes and liposomes in response to a transmembrane ammonium sulfate gradient and their cytotoxic/cytostatic activity in vitro. Anticancer Res 2001;21:2577–2583.

[32] Duangjit S, Opanasopit P, Rojanarata T, et al. Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes. J Drug Deliv 2011;2011:418316.

[33] Cevc G, Blume G. New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers. Transfersomes. Biochim Biophys Acta 2001;1514:191–205.

[34] Cevc G, Schatzlein A, Richardsen H. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. Biochim Biophys Acta 2002;1564:21–30.

[35] Erdogan M, Wright Jr JR, McAlister VC. Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-

mediated skin disorders: experimental studies in a murine model. Br J Dermatol 2002;146:964–967.
[36] Sachdeva V, Kim HD, Friden PM, et al. Iontophoresis mediated in vivo intradermal delivery of terbinafine hydrochloride. Int J Pharm 2010;393:112–118.
[37] Betz G, Imboden RI, Manidis G. Interaction of liposome formulations with human skin in vitro. Int J Pharm 2001;229:117–129.
[38] Choi MJ, Maibach HI. Elastic vesicles as topical/transdermal drug delivery systems. Int J Cosmet Sci 2005;27:211–221.
[39] Cevc G, Vierl U, Mazgareanu S. Functional characterisation of novel analgesic product based on self-regulating drug carriers. Int J Pharm 2008;360:18–28.
[40] Li G, Fan Y, Fan C, et al. Tacrolimus-loaded ethosomes: physicochemical characterization and in vivo evaluation. Eur J Pharm Biopharm 2012;82:49–57.
[41] Marsella R, Nicklin CF, Saglio S, et al. Investigation on the clinical efficacy and safety of 0.1% tacrolimus ointment (Protopic) in canine atopic dermatitis: a randomized, double-blinded, placebo-controlled, cross-over study. Vet Dermatol 2004;15:294–303.
[42] Inagaki N, Shiraishi N, Igeta K, et al. Inhibition of scratching behavior associated with allergic dermatitis in mice by tacrolimus, but not by dexamethasone. Eur J Pharmacol 2006;546:189–196.
[43] Yamamoto T, Kiyoshi N. Topical tacrolimus: an effective therapy for facial psoriasis. Eur J Dermatol 2003;13:471–473.