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Phospholipid membrane tubulation using ceramide doping "Cerosomes": Characterization and clinical application in psoriasis treatment

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ABSTRACT

Nanotechnology and material surface modification have provided a functional platform for the advancement of several medical fields such as dermatology. Furthermore, the smart choice of preparation material was proven to confer unique properties to the developed nanosystems. In this context, we focused on the sphingolipid "ceramide", whose deficiency was found to negatively affect psoriasis.

Ceramide was doped into surfactant based vesicular phospholipid systems to create tubulated vesicles "cerosomes" loaded with a model anti-psoriatic drug "tazarotene", and their properties were tested as compared to ceramide free vesicles. Cerosomes were characterized for their drug entrapment, viscosity, *in vitro* drug release, morphology, *ex vivo* drug skin deposition, thermal behavior, and were clinically tested on psoriatic patients. The factorial design study revealed that the surfactant type, the ceramide: surfactant ratio, and the presence of ethanol in the hydration buffer affected the entrapment efficiency and the viscosity of the vesicles. Ceramide increased the entrapment of tazarotene, decreased its release while enhancing its deposition within the skin, correlating with better clinical therapeutic outcome compared to the topical marketed product. Ceramide was also able to cause significant membrane tubulation in the vesicles, causing them to deviate from the conventional spherical morphology. As a conclusion, cerosomes present a new functional treatment modality for psoriasis which is worthy of future experimentation.

1. Introduction

In the past decades, topical phospholipid-based vesicular systems such as liposomes, transfersomes and ethosomes have gained interest over conventional systems in the treatment of psoriasis (Abdelgawad et al., 2016). Advantageously, vesicular systems were reported to overcome the skin's main problem represented by the stratum corneum barrier function which limits permeation of a range of molecules (El Zaafarany et al., 2010). They also possess other merits for topical delivery including prolongation of a drug's effect and minimization of drug delivery to unwanted tissues, leading to an increased bioavailability at the desired site of action (Nasr et al., 2008a; Bseiso et al., 2015; Bsieso et al., 2015; Fadel et al., 2016).

Patients suffering from psoriasis exhibit skin inflammatory reactions, making their skin different from the healthy skin. These differences are manifested by dryness due to large transepidermal water loss caused by severe depletion of ceramide, which is a main component in the skin representing about 50% of the stratum corneum (Cho et al., 2004; Nakajima et al., 2013), accompanied by the formation of painful scaly psoriatic plaques. In severe cases, patients may also exhibit skin exfoliation and peeling off (Lau et al., 2010; Badilli et al., 2011; Patil et al., 2013). Despite being major components of the epidermal layer, phospholipids were not reported to functionally ameliorate psoriasis treatment. On the other hand, ceramides were reported to positively improve psoriasis treatment through different mechanisms; they were found responsible for the maintenance of the epidermal level of water (Motta et al., 1994), they exhibit anti-proliferative effect in the epidermis during psoriasis (Lew et al., 2006), and finally, the decreased ceramide synthesis in psoriatic patients was found to positively correlate with the Psoriasis Area and Severity Index (PASI) score (Borodzicz et al., 2016).

Therefore, the aim of the current manuscript was to create a ceramide-doped vesicular system based on surfactants and phospholipids (termed "cerosomes" throughout the manuscript), loaded with a model retinoid drug; tazarotene for the treatment of psoriasis. Cerosomes were prepared at different ceramide, phospholipid and surfactant amounts, with or without ethanol as an additional permeation

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enhancer. Characterization of the prepared systems was done and they were assessed for their clinical efficacy in a pilot study comprising patients suffering from chronic plaque psoriasis.

2. Materials & methods

2.1. Materials

Absolute methanol, absolute ethanol, HPLC grade acetonitrile and chloroform were purchased from Fischer scientific (Leicestershire, United Kingdom). Acetic acid was purchased from Lobachemie (Mumbai, India). Ceramide VI was kindly provided by Evonic Company, Germany. Epikuron 200 phospholipid was kindly provided by Cargill texturizing solutions, Deutschland GmbH & Co. (Hamburg, Germany). Potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate were purchased from Adwic, El-Nasr Pharmaceutical Co. (Cairo, Egypt). Sodium deoxycholate (SDC) and tween 80 were purchased from Sigma Chemical Co. (St. Louis, USA). Spectra/Pore dialysis membrane (12.000–14.000 molecular weight cut off) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, Canada). Tazarotene was kindly provided by El Hekma Company for pharmaceuticals (Egypt). Acnitaz® gel was purchased from Marcyrl pharmaceutical company (Egypt).

2.2. Methods

2.2.1. Preparation of ceramide-doped vesicular systems "cerosomes" and control ceramide free vesicles using the thin film hydration method

Cerosomes containing tazarotene (F1–F12) were prepared using the thin film hydration method, in which the phospholipids, the drug and ceramide, with or without surfactants (Tween 80 and SDC) were dissolved in chloroform: methanol mixture (2:1, v/v) (Nasr et al., 2008a; El Zaafarany et al., 2010). The organic solvent was removed under reduced pressure using a rotary evaporator (Heidolph GmbH, model Laborota 4000 series, Germany) at 60 °C and 150 rpm till the formation of a dry lipidic film on the inner surface of the flask. The formed film was slowly hydrated for 1 h, with either 10 ml of phosphate buffer saline (pH 7.4) or hydro-alcoholic mixture of water/ethanol (30% v/v) at a ratio of (2:1), based on the composition described in (Table 1). The vesicular dispersions were sonicated for 1 h using a bath type sonicator at 40 °C (Crest ultrasonics, model 575HTAE, USA).

For comparative purposes, tazarotene loaded phospholipid based vesicles without ceramide (liposomes, transfersomes and transethosomes) were prepared according to the aforementioned method, serving as "control vesicles" for cerosomes, and were given the codes (F13–F17). Their composition is described in (Table 1).

2.2.2. Separation of unentrapped tazarotene from the prepared vesicles

In all formulations, free tazarotene was separated from the entrapped drug by ultracentrifugating the formed dispersions at 60,000 rpm at 10 °C for 30 min (El-Nesr et al., 2010) (Optima TLX, Beckman Coulter, Minnesota, USA).

2.2.3. Characterization of the prepared cerosomes and control vesicles

2.2.3.1. Determination of tazarotene entrapment efficiency EE% in vesicles. The amount of the entrapped tazarotene in both cerosomes and ceramide free vesicles was determined by diluting an aliquot of the supernatant obtained after ultracentrifugation with methanol, followed by HPLC analysis (Agilent 1100, model DE43635582, Germany) using BDS Hypersil C18 HPLC column (4.6 × 250 mm, 5 μm particle size). The mobile phase utilized was acetonitrile/water/acetic acid (65:34.5:0.5, v/v/v) at a flow rate of 2 ml/min, and the effluent was monitored at 345 nm (Nasr and Abdel-Hamid, 2016). The EE% was calculated according to the following relationship:

$$EE\% = \frac{\text{Total drug–drug in supernatant}}{\text{Total drug}} \times 100 \quad (1)$$

2.2.3.2. Viscosity measurement of vesicles. The viscosity of all vesicular formulations (cerosomes and ceramide free vesicles) was measured using a Brookfield viscometer (model DV-III, Middleboro, USA) using spindle 40 at temperature 25 °C at 100 rpm (Mouez et al., 2016).

2.2.3.3. In vitro release of tazarotene from vesicles. The release of tazarotene from all the prepared vesicles was performed using the membrane diffusion technique (Nasr et al., 2008b; Nasr et al., 2013). An amount of vesicular formulation equivalent to 1 mg tazarotene was placed in a glass cylinder of length 7 cm and diameter 2.5 cm and attached to the shaft of USP dissolution tester (Hanson Research, Chatsworth, USA) rotating at 100 rpm, and fitted with cellulose membrane at the other end. The dissolution medium was 100 ml phosphate buffer pH 7.4 containing 10% tween 80 adjusted to a temperature of 37 °C. One millimeter sample was withdrawn at predetermined time intervals (0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h) and analyzed using HPLC according to the method described in the EE% section.

Table 1
Composition and characterization of tazarotene cerosomes and control vesicles in terms of entrapment efficiency, viscosity and cumulative amount released of drug after 24 h.

Formula code*	Formula composition				EE% Mean ± S.D.	Viscosity (cp) Mean ± S.D.	Cumulative percent released after 24 h Mean ± S.D.
	Ceramide (mg)	Tween (mg)	SDC (mg)	Ethanol			
F1	112.5	12.5	–	+	89.30% ± 0.038	4.71 ± 0.01	0.3% ± 0.00
F2	87.5	37.5	–	+	69.5% ± 0.33	4.97 ± 0.01	1.42% ± 0.04
F3	62.5	62.5	–	+	62.04% ± 0.27	9.94 ± 0.4	0.1% ± 0.01
F4	112.5	12.5	–	–	98.17% ± 0.38	4.19 ± 0.15	0.49% ± 0.00
F5	87.5	37.5	–	–	95.53% ± 0.18	5.76 ± 0.15	0.64% ± 0.18
F6	62.5	62.5	–	–	93.23% ± 0.14	8.8 ± 0.7	0.35% ± 0.19
F7	112.5	–	12.5	–	99.65% ± 0.02	1.31 ± 0.15	1.75% ± 0.2
F8	87.5	–	37.5	–	97.18% ± 0.23	2.63 ± 0.7	2.39% ± 0.06
F9	62.5	–	62.5	–	92.07% ± 0.41	3.92 ± 0.01	0.46% ± 0.03
F10	112.5	–	12.5	+	95.77% ± 0.01	3.4 ± 0.69	0.1% ± 0.01
F11	87.5	–	37.5	+	90.47% ± 0.20	4.73 ± 0.35	0.16% ± 0.03
F12	62.5	–	62.5	+	73.33% ± 0.87	5.23 ± 0.52	0.53% ± 0.02
F13	–	62.5	–	–	76.3% ± 0.29	3.14 ± 0.01	29.86% ± 0.3
F14	–	62.5	–	+	58.4% ± 1.49	3.66 ± 0.15	53.29% ± 2.4
F15	–	–	62.5	–	88.1% ± 0.32	1.57 ± 0.3	0.79% ± 0.1
F16	–	–	62.5	+	70.19% ± 0.15	3.14 ± 0.15	3.2% ± 0.09
F17	–	–	–	–	98.67% ± 0.19	1.31 ± 0.15	1.78% ± 0.03

* All formulae were prepared using 10 mg tazarotene and 125 mg phospholipids.

2.2.3.4. Characterization of cerosomes based on factorial design. In order to depict how ceramide affected the behavior of surfactant-phospholipid vesicles, factorial design was utilized to study the effect of different factors on EE%, viscosity and the amount of drug released from cerosomes after 24 h. The factors under study were: the surfactant type, the ceramide: surfactant ratio, and the presence or absence of ethanol in the hydration buffer. The setup of the design is illustrated in (Table 2). The obtained factorial model was evaluated in terms of statistical significance using ANOVA. Statistical and factorial analyses were performed using Design-Expert® program v.9.0.4 (Stat-Ease Inc., Minneapolis, MN, USA).

2.2.3.5. Transmission electron microscopy (TEM) for selected cerosomal formulations and control vesicles. In order to visualize the effect of ceramide on the morphology of the vesicles, selected vesicular formulations (cerosomes and control vesicles) were examined by TEM (Nasr et al., 2008a; Bsieso et al., 2015; Fadel et al., 2016). A drop of the vesicular dispersion was placed on a copper coated grid and left for 2 min, then stained with 2% uranyl acetate solution and examined using transmission electron microscope (JEM – 100S, Joel, Tokyo, Japan).

2.2.3.6. Ex vivo skin deposition of tazarotene from selected cerosomes and control vesicles. The *ex vivo* drug deposition study was carried out in a jacketed vertical Franz diffusion apparatus (model 57-951-016, Hanson Research, Chatsworth, USA) of surface area 1.77 cm^2 and a 7 ml receptor cell volume. Full thickness dorsal rat skin samples were obtained from shaved male albino rats weighing 250–275 g after sacrificing. The skin was washed under cold running water, covered with aluminum foil and stored at −20 °C till use. On the experiment day, the skin was defrosted, hydrated in the drug release medium for 1 h before use, cut into square pieces, mounted over the diffusion cells with the stratum corneum uppermost on the space between the donor and receptor compartment, and equilibrated for 1 h (Hathout and Nasr, 2013). Phosphate buffer pH 7.4 containing 10% tween 80 was used as dissolution medium and was stirred at 100 rpm at 37 °C using a small magnetic bar. At predetermined time intervals, skin samples were carefully cleaned from excess formulations, and placed in 10 ml methanol and sonicated for 2 h to allow the extraction of tazarotene from skin layers (Nasr and Abdel-Hamid, 2016). The methanolic solutions were analyzed using HPLC as previously described.

2.2.3.7. Differential scanning calorimetry (DSC) for the selected cerosomal formulations. DSC experiments were performed with differential scanning calorimeter calibrated with indium (Perkin Elmer, model STA 6000, USA) to study the thermal behavior of cerosomes. Samples of tazarotene, ceramide, selected plain and drug loaded cerosomes were submitted to DSC analysis. The analyses were carried out on 10 mg samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate of 10 °C/min using dry nitrogen at a flow rate of (25 ml/min) (Salama et al., 2012).

2.2.3.8. Clinical efficacy of cerosomes compared to topical marketed tazarotene formulation. Twenty patients with generalized plaque psoriasis were randomly classified according to the treatment regimen given into two groups; group 1 included 10 patients receiving the selected cerosomal formula F1 in one side (A1), while group 2 included 10 patients receiving the selected cerosomal formula F6 in one side (A2) and both groups receive Acnitaz® gel in the other side (B) (Dermographic data shown in supplementary material). The research ethics committee for experimental and clinical studies at the Faculty of Pharmacy, Ain Shams University approved the clinical experiment (REC ASU-65) with the principles outlined in the declaration of Helsinki for human subject experimentation being followed. Inclusion criteria for the pilot clinical trial were patients with bilateral symmetrical psoriasis plaques who hadn't received any topical or systemic antipsoriatic treatment or phototherapy for at least three months before the beginning of the study. Exclusion criteria involved patients with erythrodermic or pustular psoriasis. All patients were subjected to full history taking; personal history (age, sex and occupation), present history (onset and duration) and past history (previous medications) in addition to family history. Clinical and dermatological examinations were conducted for each patient, followed by written consent taking. Patients were photographed before treatment with follow up photographing up to 8 weeks.

Patients were instructed to apply a thin film of either cerosomal formulation incorporated in gel at one side (A1 or A2) and Acnitaz® gel in the other side (B) for 8 weeks. Patients enrolled for the study were not permitted to concomitantly use any antipsoriatic drug other than the trial formulations or the other comparative topical medication (Acnitaz® gel). Furthermore, they were asked to report any discomfort or irritation encountered during the study.

The severity of psoriasis was evaluated using the PASI score (Psoriasis Area and Severity Index) according to Fredriksson and Pettersson, 1978. To determine PASI score, four body areas are included:

(1) The head (H) corresponding to 10% of the body surface area (BSA).

(2) The trunk (T) corresponding to 30% of BSA.

(3) The upper extremities (U) corresponding to 20% of BSA.

(4) The lower extremities (L) corresponding to 40% of BSA.

According to the extent of the lesions, each area of psoriatic involvement (A) is to be assigned a numerical value within the range of (0–6) according to the following scale:

0 = non, 1 ≤ 10%, 2 ≥ 10%–30%, 3 ≥ 30%–50%, 4 ≥ 50%–70%, 5 ≥ 70%–90%, 6 ≥ 90%–100%.

In order to evaluate the severity of the psoriatic lesions, three target symptoms, namely erythema (E), infiltration (I) and desquamation (D) were also assessed on a (0–4) scale as follows: 0 = non, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe. To calculate the PASI score, the sum of the severity rating for these 3 main changes (E, I and D) was multiplied with a numerical value of the areas involved and with the various percentages of the four body areas. The formula for calculating PASI score was compiled as follows:

$$\begin{aligned} PASI = {} & [(EH + IH + DH) \times AH] \times 0.1 \\ & + [(ET + IT + DT) \times AT] \times 0.3 \\ & + [(EU + IU + DU) \times AU] \times 0.2 \\ & + [(EL + IL + DL) \times AL] \times 0.4 \end{aligned} \quad (2)$$

Accordingly, the severity is considered as: mild if PASI score is <15, moderate if PASI score = 15–25 or severe if PASI score is >25.

Table 2
Setup of the optimal custom factorially designed experiment.

Factor	Name	Type	Number of levels	Level codes and designations
A	Ceramide: surfactant ratio	Numeric	3	−1 (9:1) 0 (2.3:1) 1 (1:1)
B	Type of surfactant	Categoric	2	Level 1 of B (Tween 80) Level 2 of B (SDC)
C	Presence of ethanol	Categoric	2	Level 1 of C (Ethanol presence) Level 2 of C (Ethanol absence)

2.2.3.9. Statistical analysis of data. All the presented data were reported as the mean with standard deviation (S.D) of triplicate individual batches, followed by comparison using (ANOVA) followed by Tukey Kraumer multiple comparison test using Graph Pad Instat® software. The statistical significance level (p) value was set at ≤0.05. For clinical experimentation, PASI scores were statistically compared before and after treatment for the same lesion within the same group using Wilcoxon matched pairs test at $p \leq 0.05$. Mann-Whitney test was used to assess the statistical significance between lesion A and B of the same group at $p \leq 0.05$.

3. Results

3.1. Determination of tazarotene EE% in vesicles

As shown in (Table 1), the EE% of tazarotene in cerosomes ranged from 62.04% to 99.65%. The equation modeling the effect of different factors on EE% of tazarotene in cerosomes was:

$$Y = 88.27 + 7.93A + 3.7B + 7.78C - 0.42AB - 4.8\,AC - 3.27BC \quad (3)$$

in which Y represents the response (EE %), A: effect of ceramide: surfactant ratio, B: effect of surfactant type and C: effect of ethanol presence. The 2 factor interaction model (2FI) was selected as the best fit model for the data, with R^2 of 0.9666, adjusted R^2 of 0.9484 and predictive R^2 of 0.8987. The summary of the ANOVA analysis of the model is tabulated in (Table 3), and the cube graph clarifying the effects of the aforementioned 3 factors is illustrated in (Fig. 1).

It was observed that increasing the ceramide amount from 62.5 mg to 112.5 mg (increasing the ceramide/edge activator ratio from 1:1 to 9:1) significantly increased the EE% of tazarotene regardless the type of edge activator or the presence/absence of ethanol ($P < 0.05$). Cerosomes prepared using sodium deoxycholate had mostly higher EE% values for tazarotene compared to those prepared using tween 80 as edge activator ($P < 0.05$). This was also confirmed by the factorial design model which indicated a positive correlation between EE% of cerosomes and factor B (type of surfactant). The effect of SDC based cerosomes being superior in EE% to their tween 80 based counterparts is more observed in presence of ethanol than in absence of ethanol, as also confirmed by the significant value of the 2 factor interactions (BC). It can also be deduced from (Fig. 1) that the addition of ethanol to the hydrating buffer significantly decreased the tazarotene EE% compared to the formulations prepared without ethanol ($P < 0.05$). The ability of ethanol to decrease EE% of tazarotene was more observant in tween 80 cerosomes, as also confirmed from the significant 2 factor interaction (BC) of the factorial model.

As also evident in (Table 1), tazarotene was encapsulated in the ceramide free control vesicles in the following order: liposomes ˃ SDC based transfersomes ˃ tween 80 based transfersomes ˃ SDC based transethosomes ˃ tween 80 based transethosomes.

Table 3
Summary of ANOVA analysis of the model derived from the factorial study determining the significance of the variables on EE% of cerosomes.

	Sum of squares	d.f.	Mean square	F value	*P* value
Model	2779.78	6	463.30	53.06	<0.0001
A	586.29	1	586.29	67.15	<0.0001
B	239.44	1	239.44	27.42	0.0003
C	1056.16	1	1056.16	120.96	<0.0001
AB	1.65	1	1.65	0.19	0.6720
AC	214.66	1	214.66	24.58	0.0004
BC	187.21	1	187.21	21.44	0.0007
Pure error	0.1	6	0.017		

3.2. Viscosity measurement of cerosomes and control vesicles

Upon measuring the viscosity of tazarotene cerosomes, the prepared formulations showed viscosity values ranging from 1.31 to 9.94 cp as shown in (Table 1). The equation describing the model relating the effect of factors A, B and C to viscosity was quadratic, and is represented by the following equation:

$$Y = 4.52 - 1.77A - 1.33B - 0.53C + 0.66AB - 0.032AC - 0.42BC + 0.76A^2 \quad (4)$$

The model R^2 was 0.9419, the adjusted R^2 was 0.9013, and predictive R^2 was 0.7866. The factors were same as those studied for EE%, and their effect was illustrated in (Fig. 2), and the summary of the ANOVA analysis of the model is shown in (Table 4).

It was obvious that increasing the amount of tween 80 or SDC from 12.5 mg to 62.5 mg and decreasing the amount of ceramide from 112.5 mg to 62.5 mg significantly increased the viscosity of the formulations ($P < 0.05$). Tween 80 based vesicles exhibited significantly higher viscosity their SDC counterparts ($P < 0.05$), suggesting that the edge activator amount was the key factor influencing the viscosity of the vesicles. The presence of ethanol in the formulations generally increased their viscosity more than their counterparts formulated without ethanol, with this increase being statistically significant in most cases ($P < 0.05$).

The viscosity of ceramide free control vesicles ranged from 1.31–3.36 cp, which were smaller in values compared to the cerosomes viscosity range. The addition of ceramide increased the viscosity of tween 80 control vesicles with higher increments than SDC vesicles regardless of the presence or absence of ethanol, reflecting the importance of the effect of type of surfactant on viscosity. As also obtained with cerosomal formulations, tween 80 control vesicles exhibited significantly higher viscosity their SDC counterparts ($P < 0.05$). Moreover, transethosomes showed significantly higher viscosity compared to their transfersomal counterparts ($P < 0.05$).

3.3. In vitro drug release studies

In vitro release studies conducted on tazarotene vesicles showed that the cumulative percentage of tazarotene released after 24 h was quite low (0.1%–2.39%), as shown in (Table 1), with the release profile found to follow Higuchi diffusion kinetics. As the results were close to each other, they were excluded from characterization through the factorial design. On the other hand, the ceramide free control vesicles containing tween 80 (F13, F14) showed high *in vitro* drug release (29.86% and 53.29%) respectively.

3.4. Transmission electron microscopy (TEM) for selected cerosomes formulations and control vesicles

For TEM examination, formulations F1 and F6 were selected as representatives of tween 80 cerosomes with high and low ceramide contents respectively, while F9, F10 were selected as their SDC counterparts. TEM showed dramatic change in the shape of vesicles upon ceramide incorporation within vesicles. For liposomal (F17), transfersomal (F13 and F15) and transethosomal control vesicles (F12 and F16) which are free from ceramide, all showed the traditionally known shape of spherical vesicles as displayed in (Fig. 3). On the other hand, cerosomal formulations showed fiber-like morphology with the formation of elongated intertwined ceramide tubules in both tween 80 and SDC cerosomes as shown in (Figs. 4 and 5) respectively. Careful inspection of the micrographs showed the presence of spherical vesicles along with the tubular vesicles with SDC cerosomes.

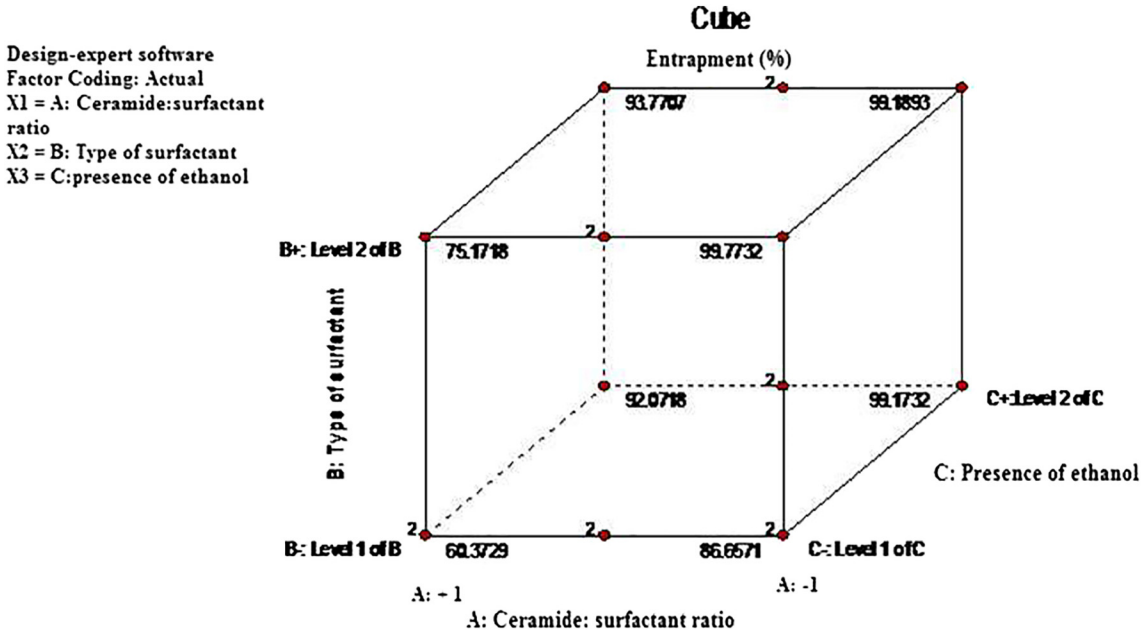

Fig. 1. Effect of ceramide: surfactant ratio, type of surfactant and presence of ethanol on the EE% of tazarotene cerosomes.

3.5. Ex vivo skin deposition of selected tazarotene cerosomes and ceramide free vesicles

Since tween 80 cerosomes F1 and F6 displayed higher incidence of tubular morphology than SDC formulations F9 and F10, they were selected for the conduction of *ex-vivo* skin deposition experiments. For comparative purposes, formulations F13 and F14 which displayed high *in vitro* release were tested for their *ex-vivo* deposition as well. As shown in (Table 5), the cerosomal formulations showed significantly higher drug skin deposition after application for 24 to 48 to 72 h compared to the marketed product ($P < 0.05$), reaching 69.62% and 58.5% for F1 and F6 respectively, while the ceramide free control vesicles F13 and F14 showed very low deposition percentages not exceeding 2% in the skin, which were significantly lower than the marketed product Acnitaz® ($P < 0.05$).

3.6. Differential scanning calorimetry (DSC) for the selected cerosomal formulations

As illustrated in (Fig. 6), the tazarotene melting peak was found to completely disappear in cerosomal formulations.

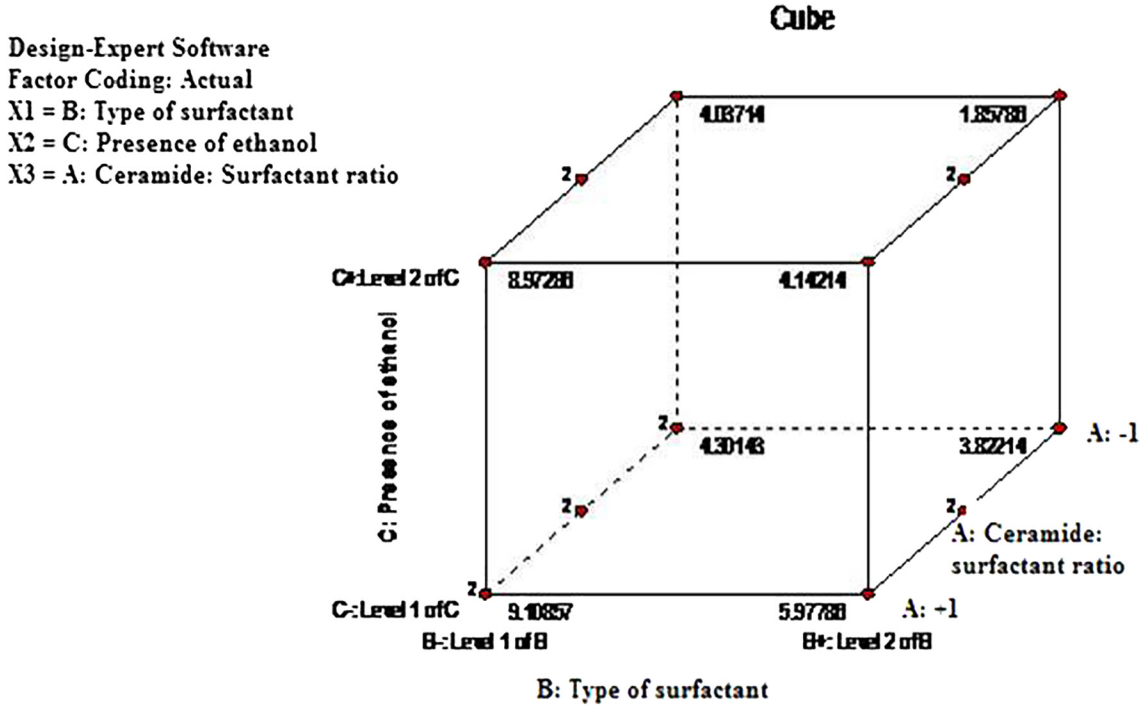

Fig. 2. Effect of ceramide: surfactant ratio, type of surfactant and presence of ethanol on the viscosity of tazarotene cerosomes.

Table 4
Summary of ANOVA analysis of the model derived from the factorial study determining the significance of the variables on viscosity of cerosomes.

	Sum of squares	d.f.	Mean square	F value	*P* value
Model	83.79	7	11.97	23.18	<0.0001
A	29.33	1	29.33	56.80	<0.0001
B	30.55	1	30.55	59.15	<0.0001
C	4.78	1	4.78	9.25	0.0124
AB	4.10	1	4.10	7.94	0.0182
AC	9.643E-003	1	9.643E − 003	0.019	0.8940
BC	3.13	1	3.13	6.06	0.0335
A^2	2.47	1	2.47	4.78	0.0536
Pure error	0.14	6	0.023		

3.7. Clinical efficacy of cerosomes compared to topical marketed tazarotene formulation

(Tables 6 and 7) display the PASI scores for patients before and after treatment with either cerosomal formulation F1, F6 or Acnitaz® marketed gel. As evident in (Table 6) for group 1 treated with formula F1/Acnitaz® gel, the mean PASI scores for lesion A1 treated with formula F1 before and after treatment were 14.85 ± 4.54 and 5.13 ± 2.1 respectively; showing significant lesion reduction after 8 weeks treatment with formula F1 ($P < 0.05$). On the other hand, the mean PASI scores for lesion B treated with Acnitaz® gel before and after treatment were 14.07 ± 4.38 and 9.28 ± 2.73 respectively, also showing significant reduction after 8 weeks of treatment ($P < 0.05$), but with better outcome with formula F1. Mann-Whitney non parametric test showed that PASI scores for patients lesions treated with formula F1 (A1) were significantly lower than those treated with Acnitaz® gel (B) ($P < 0.05$). (Fig. 7) represents a male patient suffering from plaque psoriasis in the forearms, in which lesion A1 was treated with formula F1 and lesion B was treated with Acnitaz® gel showing marked improvement with the former and moderate improvement with the latter.

Regarding group 2 treated with formula F6/Acnitaz® gel, the mean PASI scores for lesions A2 treated with formula F6 before and after treatment were 12.68 ± 3.32 and 8.91 ± 2.67 respectively; showing significant lesion reduction after 8 weeks of treatment ($P < 0.05$). On the other hand, the mean PASI scores for lesions B treated with Acnitaz® gel before and after treatment were 11.87 ± 3.46 and 8.23 ± 2.81 respectively, also showing significant reduction ($P < 0.05$). No statistically significant difference was obtained in PASI scores between cerosomal formula F6 and Acnitaz® gel, as also demonstrated in (Fig. 8).

4. Discussion

Ceramides are the simplest, the least polar and the most hydrophobic type of sphingolipids, which are responsible for the barrier function of the stratum corneum (Khazanov et al., 2008). Ceramides represent 50% lipid weight of the stratum corneum, but are present in much lower proportion in cell membranes (Xu et al., 2009). It was observed that patients suffering from psoriasis exhibit depletion in ceramide level in skin compared to healthy individuals. This leads to increase in transepithelial water loss causing dryness and inflammation of the skin (Cho et al., 2004). Ceramide VI which is used for vesicular preparation in the current manuscript is a synthetic ceramide consisting of a phytosphingosine base backbone acylated with a long chain alpha-hydroxy stearic acid, in which both are saturated. It has the same stereochemical configuration as human skin's ceramide with a small difference that its phytosphingosine base and fatty acids are saturated.

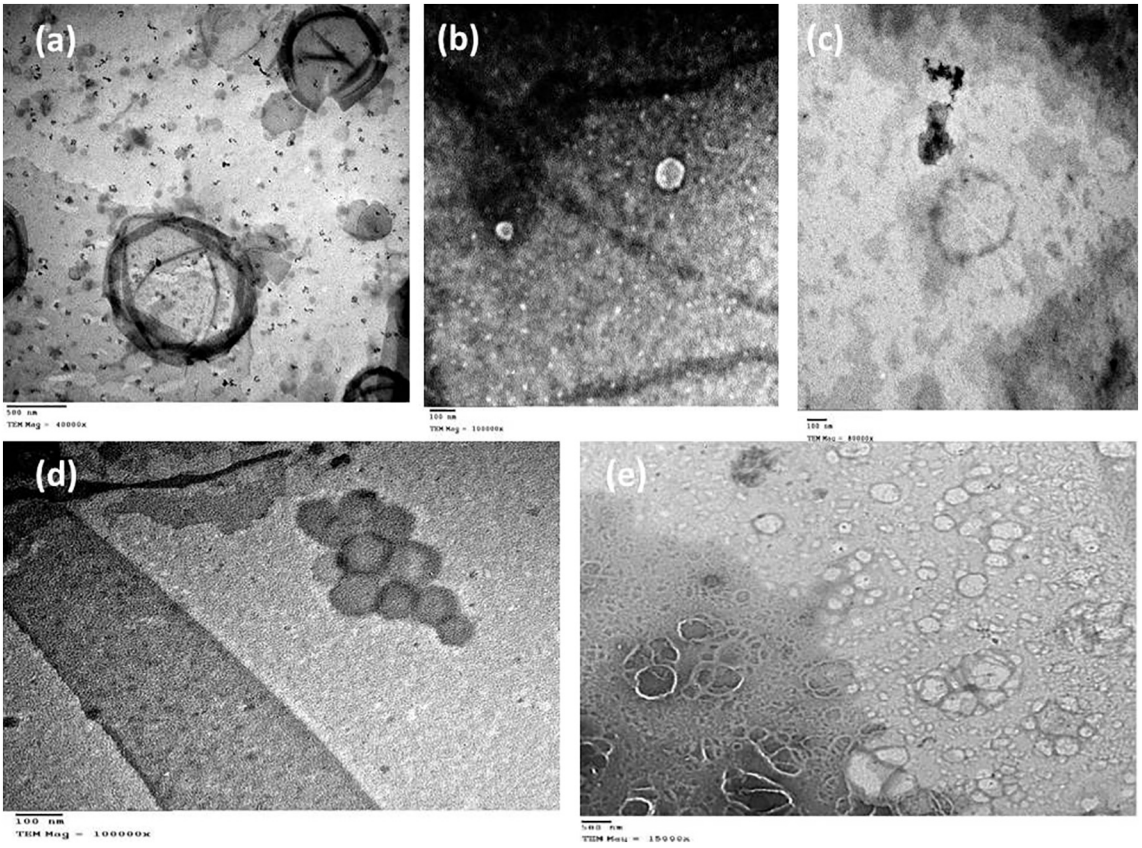

Fig. 3. Transmission electron micrographs of ceramide free vesicles (a) liposomes formula F17 at 40000× (b) SDC transethosomes formula F16 at 100000× (c) tween 80 transethosomes formula F14 at 80000× (d) tween 80 transfersomes formula F13 at 100000× (e) SDC transfersomes formula F15 at 15000×.

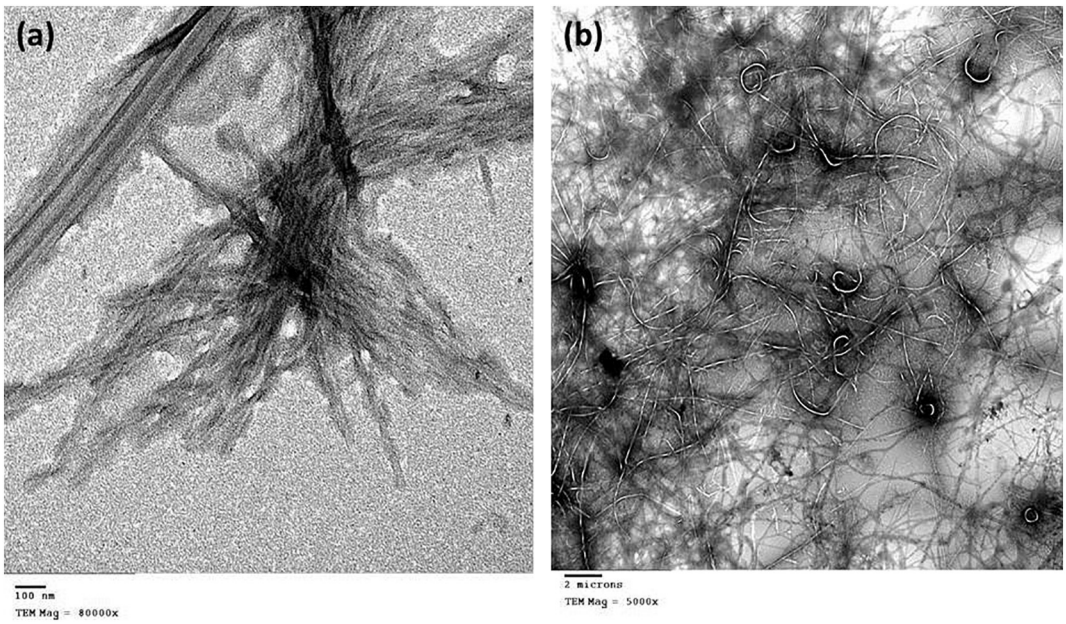

Fig. 4. Transmission electron micrographs of tween 80 cerosomal formulations (a) F1 containing high ceramide content at 80000× (b) F6 containing low ceramide content at 5000×.

The structural similarity of synthetic ceramide VI to natural skin lipid "ceramide 7" facilitates its penetration with active drugs through the stratum corneum through the perturbation of intercellular lipid organization (Xu et al., 2009). Therefore, the present manuscript examined the possibility of ceramide "doping" within one of the well-established delivery systems (the vesicular delivery systems), and how this affected the vesicles in terms of physicochemical properties, skin deposition, and clinical potential in psoriatic patients. A model drug; tazarotene, was utilized as the anti-psoriatic drug of choice to be incorporated in our system as it is a hydrophobic drug of log P 5.96, making it a suitable candidate for incorporation within our lipidic system, while being an approved retinoid by the FDA for topical delivery of psoriasis (Guenther, 2002).

Upon using ceramide as the sole lipid for vesicle formation, the formulations showed precipitation at the first instant of buffer addition, regardless the presence or absence of surfactants (data not shown) owing to the ceramide's very small polar head group and its significant hydrophobicity (Khazanov et al., 2008). This came in accordance with other authors (Xu et al., 2009; Park et al., 2013) who reported that the presence of phosphatidylcholine facilitated the incorporation of ceramide into vesicular bilayers. However, when attempting to prepare cerosomes without surfactants or alcohol, aggregates were formed during the 1 h hydration period of the vesicles (data not shown); which suggested the necessity of the surfactant presence in order to yield sufficiently stable vesicles formulated using this double lipidic mixture (phosphatidylcholine-ceramide). Therefore, formulations F1–F12 were prepared using two surfactant types; tween 80 and SDC, in presence or absence of ethanol as an additional penetration enhancer.

Delineating some facts from the factorial models of highest reliability (Nasr et al., 2011), it was evident that the increase in EE% of tazarotene was closely related to the increase in ceramide content, which may be explained on the basis of the hydrophobicity of ceramide,

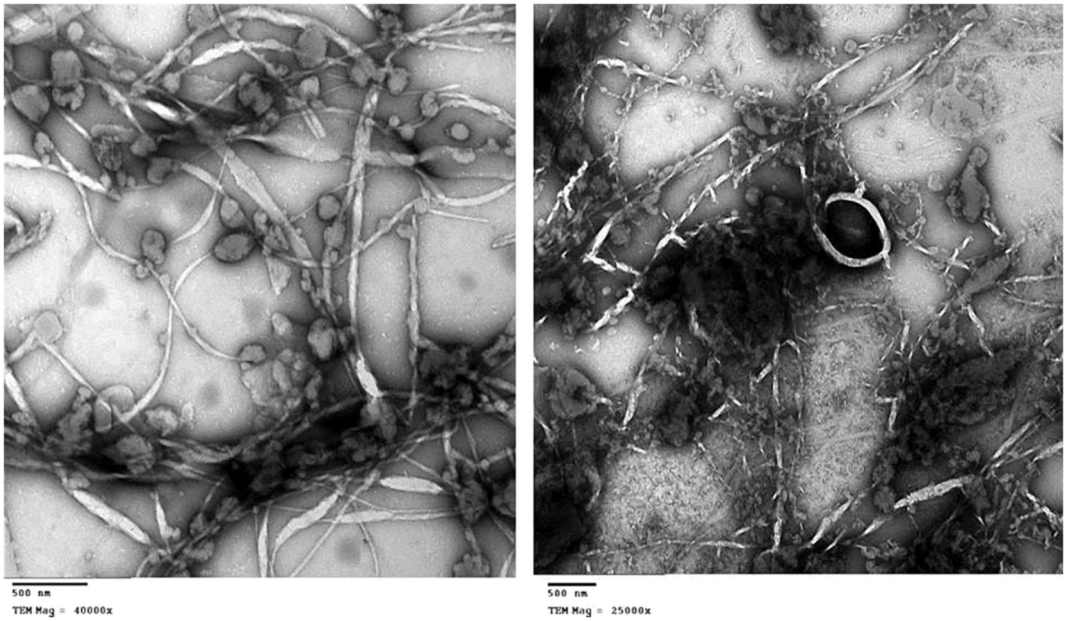

Fig. 5. Transmission electron micrographs of SDC cerosomal formulations (a) F10 containing high ceramide content at 40000× (b) F9 containing low ceramide content at 25000×.

Table 5
Percentages of tazarotene retained in rat skin at different time intervals up to 72 h for cerosomal formulations compared to control vesicles and the marketed product Acnitaz®.

Formula code	% tazarotene retained after 24 h	% tazarotene retained after 48 h	% tazarotene retained after 72 h
F1	50.36% ± 1.3	68.77% ± 1.72	69.62% ± 2.3
F6	50.93% ± 0.4	62.37% ± 0.45	58.5% ± 0.4
F13	1.72% ± 0.24	1.5% ± 0.028	1.22% ± 0.21
F14	1.3% ± 0.28	0.7% ± 0.14	0.6% ± 0.14
Acnitaz ® gel	35.11% ± 1.72	27.94% ± 2.98	22.27% ± 1.58

causing better incorporation of the lipophilic tazarotene within vesicles (Xu et al., 2009). The fact that SDC cerosomes displayed higher EE% than tween 80 cerosomes may be ascribed to the superior solubilizing effect of tween 80 on tazarotene, causing an increased fraction of tazarotene solubilized in the aqueous phase and separated from the pellet by ultracentrifugation. Similar results were obtained with Maurya et al., 2010 who reported that vesicles prepared using SDC showed higher EE% of indinavir compared to those prepared using tween 80. Another finding deduced from the factorial model was that the presence of ethanol decreased tazarotene EE%, which may be attributed to the presence of ethanol fractions within the aqueous phase in which the vesicles are dispersed, leading to a consequent solubilization of tazarotene in the supernatant. It could also be attributed to the increased permeability of the vesicular membrane caused by ethanol, consequently leading to drug leakage (Shamma and Elsayed, 2013). Those three facts (decreased EE% of tazarotene by ceramide absence, by using tween 80 as surfactant during preparation, and by the presence of ethanol) were further confirmed when studying the EE% of control vesicles.

Despite the reported effect of ceramide in increasing the viscosity due to formation of gel like domains (Sot et al., 2009) and increasing the microviscosity of bilayers (Imura et al., 2001), it was evident that the surfactant amount was the key factor influencing the viscosity of the vesicles. This may be ascribed to the high viscosity of tween 80 itself (605 cp) and the possible binding interaction between the bile salt and the phospholipid, resulting in viscous solutions (Cheng et al., 2014). The high viscosity of tween 80 also accounts for the higher viscosity of tween 80 cerosomes compared to their SDC counterparts. The presence of ethanol in the cerosomal formulations generally increased their viscosity owing to the formation of gel domains in the phospholipid bilayers (Bseiso et al., 2016).

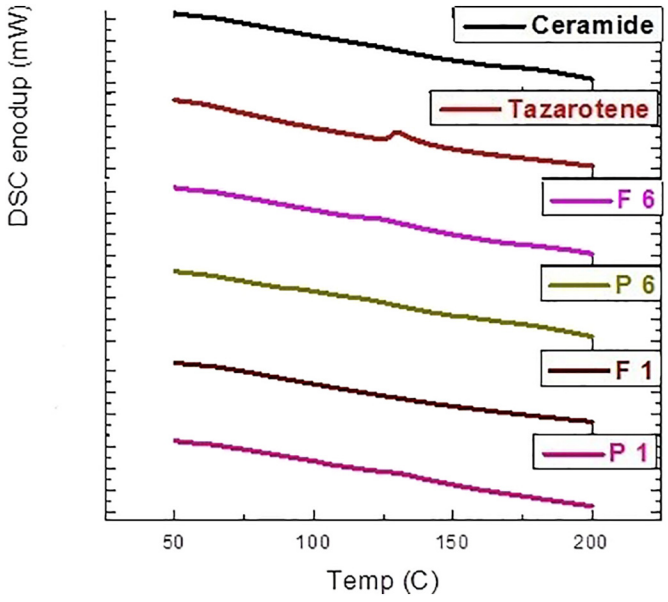

Fig. 6. DSC thermograms of tazarotene, ceramide, cerosomal formulations F1 and F6 and their blank counterparts P1 and P6.

Table 6
PASI scores for group 1 patients before and after treatment with cerosomal formulation F1 on lesion (A1) and Acnitaz® gel on lesion (B).

Case number	PASI score			
	Lesion A1		Lesion B	
	Before treatment	8 weeks after treatment	Before treatment	8 weeks after treatment
1	19.2	5.4	18.3	10
2	12.3	6.9	11.9	11
3	10.8	4.7	10.5	8.91
4	5.7	3.1	4.9	2.1
5	18.9	6.4	16.8	10.2
6	20.3	8.2	19.4	11.9
7	13	2.5	11.9	8.8
8	18	5.4	17.5	11.1
9	15.3	6.9	14.9	9.9
10	15	1.8	14.6	8.9

Upon comparing cerosomes to the control vesicles, it was clear that cerosomes displayed significantly higher viscosity than the latter ($P < 0.05$), which could be attributed to the effect of ceramide on increasing the microviscosity of the vesicular bilayer membranes (Imura et al., 2001).

The *in vitro* release of tazarotene was influenced by the lipidic components represented by phosphatidylcholine and ceramide, being responsible for the hydrophobicity of the vesicles, leading to a concomitant increase in affinity to the hydrophobic tazarotene to the lipidic vesicular components and its minimal release. Tween 80 caused a more fluidizing effect in the lipidic membrane compared to SDC, leading to an increased amount of tazarotene permeated across the dialysis membrane owing to its highly flexible and non-bulky hydrocarbon chains (Chen et al., 2013).

Despite the low overall release of tazarotene from cerosomes, this was not considered a disadvantage in our case, since the objective of this work was to strengthen the barrier function of diseased, affected and/or aged skin by administering ceramide into the stratum corneum, and to investigate the deposition of the systems rather than their permeation.

TEM examination showed that the addition of ceramide to the composition of the vesicles resulted in a dramatic membrane shape evolution from the spherical to the tubular morphology. In all formulations containing ceramide "cerosomes", the predominant morphology was that of elongated intertwined ceramide tubules, with the less frequent existence of vesicles. This was also reported by Xu et al., 2009 who noted that doping of phosphatidylcholine with ceramide caused elongation of their prepared vesicles attributed to the partitioning of ceramide

Table 7
PASI scores for group 2 patients before and after treatment with cerosomal formulation F6 on lesion (A2) and Acnitaz® gel on lesion (B).

Case number	PASI score			
	Lesion A2		Lesion B	
	Before treatment	8 weeks after treatment	Before treatment	8 weeks after treatment
1	18.2	11.3	17.9	13
2	13.3	10.9	11.2	7
3	11.2	7.7	11	9.2
4	5.7	3.1	4.8	4
5	14.9	9.4	13.8	10
6	15.3	12.2	14.7	10.9
7	13	8.6	12.9	5.5
8	11.1	6.4	10	5.8
9	13.1	9.2	12.4	9.9
10	11	10.3	10	7

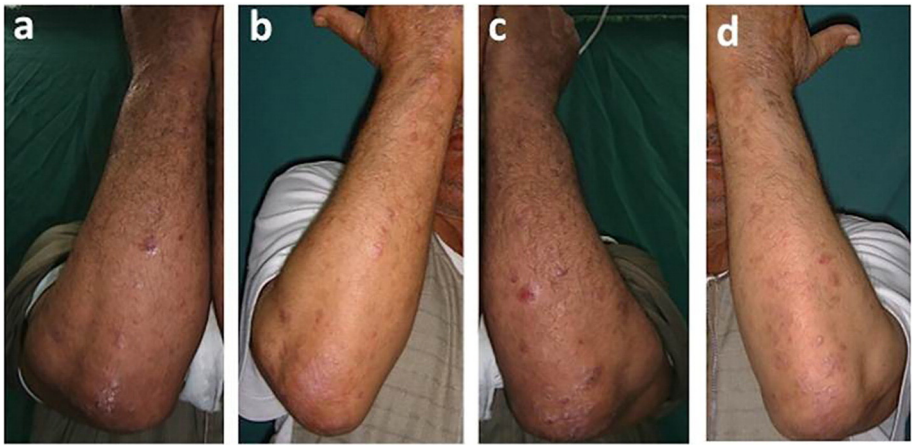

Fig. 7. Male patient presenting with plaque psoriasis over right (a) and left (c) forearms. There was marked improvement after 8 weeks of treatment with formula F1 on lesion A1 on right forearm (b) and moderate improvement with Acnitaz® gel on lesion B on left forearm (d).

VI into the phospholipid bilayer accompanied by rigidification of the interface. The high packing parameter of ceramide (1.2) compared to that of the phosphatidylcholine (0.7) caused flattening of the phospholipid bilayer curvature upon hydration with aqueous buffer. The occasional presence of spherical vesicles along with the tubules (resulting in a characteristic bulbous feature of the tubules) as encountered in formula F10 confirmed another observation by the same authors who reported that ceramide VI tends to distribute in a non-uniform pattern in the bilayer, resulting in ceramide rich domains with a flat morphology and ceramide poor domains with spherical morphology. The existence of ceramide poor domains was more obvious with SDC cerosomes, hence, tween 80 cerosomes proceeded to the *ex vivo* skin deposition experiment.

All cerosomal formulations were superior in terms of the amount of drug deposited in the skin compared to the marketed product Acnitaz® ($P < 0.05$) and transfersomal and transethosomal formulations F13 and F14, suggesting the role of ceramide in enhancing the deposition of formulations within the skin. Worthy to note that formulations F13 and F14 displayed the highest percentages of drug release in the *in vitro* release experiments, suggesting that their poor *ex vivo* skin retention

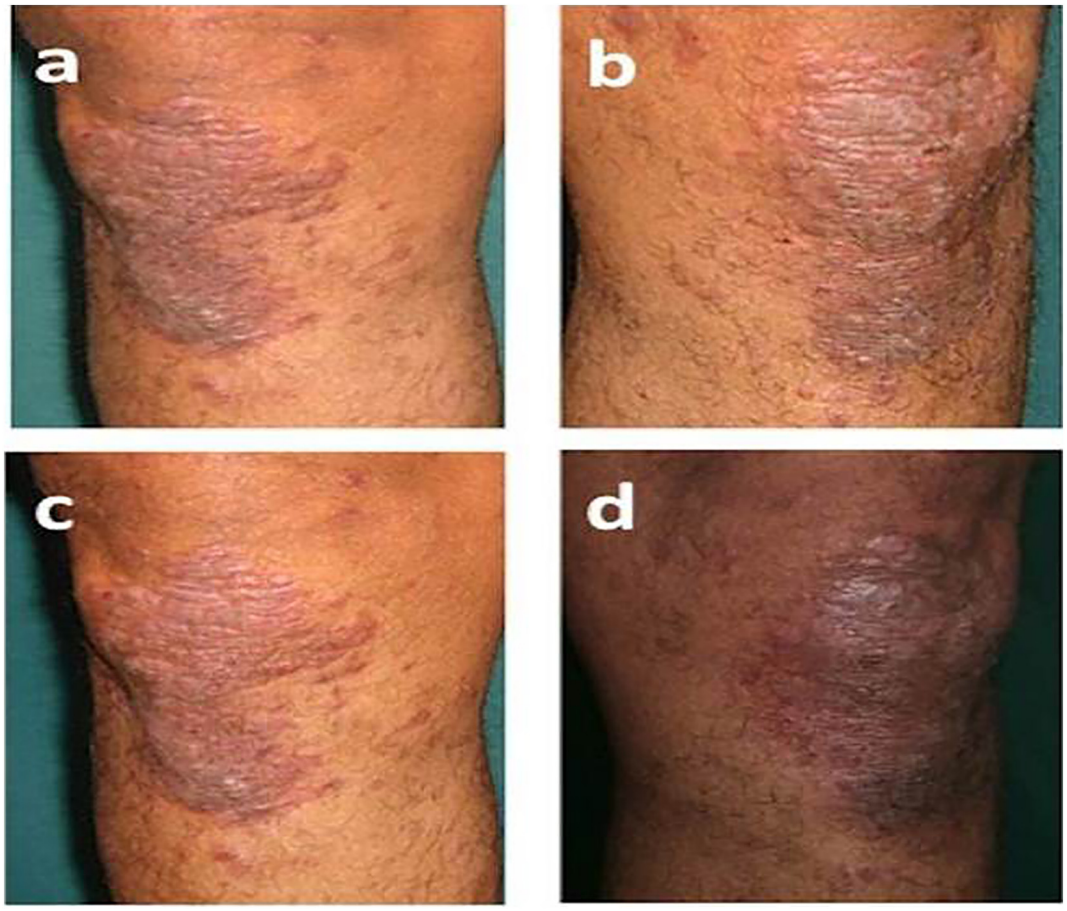

Fig. 8. Male patient presenting with plaque psoriasis over right (a) and left (b) knees. There was mild improvement after 8 weeks of treatment with both B on right knee (c) and formula F6 A2 on left knee (d).

ability is probably attributed to their high permeation into the receptor compartment. Acnitaz® marketed product showed a slight decrease in the amount of tazarotene retained with time which may be attributed to the possible degradation of tazarotene with time when being administered in topical conventional formulations (Hecker et al., 2000).

The superior deposition encountered with cerosomes could be ascribed to the fact that ceramide VI was reported to interact with the keratin of the corneocytes, demonstrating lipid bilayer holding ability and high fusion activity to the skin leading to drug localization (Corbe et al., 2007; Tokudome et al., 2009; Ochalek et al., 2012; Gaur et al., 2014). The enrichment of the stratum corneum with ceramide VI from our prepared cerosomes resulted in enhanced skin deposition rather than transdermal permeation of tazarotene. The solubilization of tazarotene within cerosomes was confirmed by the disappearance of the tazarotene melting peak upon performing DSC.

As previously reported in clinical trials of psoriasis, 50% improvement of PASI is considered a clinically meaningful endpoint indicative of success of the trial (Carlin et al., 2004; Feldman and Krueger, 2005; Nasr et al., 2016). Therefore, despite the statistically significant reduction in PASI scores for both formula F1 and Acnitaz® gel before and after treatment, formula F1 led to approximately 65% reduction in the mean PASI score after 8 weeks of treatment, while Acnitaz® gel only led to 34% reduction in the mean PASI score, proving its superiority to the conventional marketed product Acnitaz® in achieving therapeutic efficacy for psoriasis patients. On the other hand, both formulations F6 and Acnitaz® gel led to approximately 30% reduction in the mean PASI score after 8 weeks of treatment, suggesting the comparable therapeutic effect of formula F6 to the marketed Acnitaz® gel. The cerosomal formula F1 containing ethanol and high amount of ceramide was superior in providing clinical efficacy in psoriatic patients, which may either be attributed to its significantly higher deposition potential than F6 as shown in the *ex vivo* skin deposition study, or to the presence of ethanol which might have facilitated the penetration of tazarotene through the skin by acting as skin permeation enhancer (Lachenmeier, 2008). Another merit of ceramide containing vesicles compared to Acnitaz® gel is that in all patients, lesions A1 and A2 were lacking signs of skin sensitization, with patients reporting no burning sensation upon application that was usually encountered upon application of the conventional Acnitaz® gel on lesion B. This suggests that the encapsulation of tazarotene (which is known to be irritant to the skin) within vesicles was able to reduce its irritation potential.

5. Conclusion

The advancement in drug delivery technologies has led to the enhancement of therapeutic efficacy of many drugs. In the current manuscript, the utilization of a sphingolipid; "ceramide" within the regular phospholipid vesicles was proven to confer formulation advantages to the system as well as achieve enhanced clinical efficacy. With more ongoing research on the clinical perspective of efficacy of different nanosystems, there will be more innovations in this regard, either through the utilization of new materials, exploration of new drugs, or development of new delivery systems. The authors believe that creation of composite systems containing functional additives "ceramide in our case" would allow emerging of new delivery systems with exciting properties. An interesting point worthy of futuristic research is to use the cerosomes described in the current manuscript for delivery of drugs which require high skin deposition to exert their therapeutic effect, taking advantage of the nanofibrous nature of cerosomes and their lipidic content.

Declaration of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejps.2017.02.030.

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