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Original Article

Improvement of dermal delivery of tetracycline using vesicular nanostructures

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Abstract

The objective of this investigation was to study the potential use of nanoliposomes and nanotransfersomes in dermal delivery of tetracycline hydrochloride (TC) for acne treatment. Vesicular nanostructures were prepared by thin film hydration method and evaluated for their size, zeta potential, morphology, and entrapment efficiency. Minimal inhibitory concentration values of TC-loaded vesicles were evaluated and compared with TC aqueous solution against *Staphylococcus epidermis*. *In vitro* drug release and *ex vivo* drug permeation through the excised rat skin were performed to assess drug delivery efficiency. Particle size, zeta potential, and entrapment efficiency of prepared nanoliposomes and nanotransfersomes were found to be 75 and 78 nm, 17 and 7 mV, and 45 and 55%, respectively. Antimicrobial analysis indicated that there was no difference between vesicular formulations and aqueous solution of TC. *In vitro* drug release study indicated that nanoliposomes could release TC 2.6 folds more than nanotransfersomes, and skin permeation study showed that the permeability of TC-loaded nanotransfersomes was 1.6 times higher than nanoliposomes which was also confirmed by fluorescence microscope imaging. These findings concluded that nanoliposomal and especially nanotransfersomal formulations could be proposed as the potential approach for better therapeutic performance of TC against acne.

Keywords: Acne; Dermal drug delivery; Liposome; Nanoparticle; Tetracycline; Transferosome.

INTRODUCTION

In spite of tremendous advantages of dermal drug delivery (1), stratum corneum (SC), the outermost layer of skin consisting of corneocytes surrounded by lipid bilayers, is the main barrier for drug delivery into skin. To overcome this problem, nanoparticles have been introduced for improvement of dermal drug delivery (2). Acne vulgaris is an inflammation of the sebaceous glands and appendices with the immune response to various gram-positive bacteria, usually *Staphylococcus epidermidis* (S. *epidermis*) which colonize in sebum rich follicles. Therefore, medicines should be reached to the epidermis to inhibit bacteria growth. Almost 85% of people between the ages of 12 and 25 years suffer from some degree of this disorder (3). Oral delivery of tetracycline hydrochloride (TC), an effective antibiotic in acne therapy has shown obvious side effects such as vomiting, kidney damage, cramps, diarrhea, and reduced effectiveness following the administration of milk or antacid drugs, which may reduce the efficacy of the treatment and deteriorate patient compliance (4). Therefore, dermal TC delivery is a step forward to remove the obstacles for its oral administration to acne treatment.

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However, TC is a water-soluble (freely soluble in water) drug commonly in the dissociated form at the physiological pH which limits the capacity of TC for passive penetration into skin layers (5). The drug carrier selection is one of the most important entities essential for successful targeted drug delivery. The suitable characteristics of carriers for skin delivery are high capacity for drug loading, biocompatibility, low systemic absorption, and high dermal accumulation (6,7). Lipid-based nanoparticles not only improve skin absorption but also can permit drug targeting to the skin layers and skin accumulation. Among the lipid based nanoparticles, lipid vesicular systems are more promising due to the capability to carry both hydrophobic and hydrophilic drugs because of bilayer structure, non-toxic and biodegradable characteristics as a result of the presence of phospholipid in its composition, easy to scale up for industrial manufacturing, and skin localization of drugs (8). The system enhances drug penetration into skin by diffusing into the SC, disrupting its bilayer fluidity, loosening the lipid structure of the SC and providing impaired barrier function of this layer of skin to the drug (9). Moreover, some studies reported that phospholipids may mix with the SC lipids creating a lipid-enriched environment accelerating drug absorption (10,11). A new field of research in topical delivery field was opened with the use of liposomes for the dermal delivery and since then a wide range of novel lipid-based vesicles such as deformable liposomes, which are currently known as transfersomes have been developed (12,13). Addition of non-ionic surfactants to the liposomal bilayer structure provides the required flexibility to liposomes and this new structure named as transfersome (8,13). The aim of this study was to enhance dermal delivery of TC using liposomal and transfersomal formulations to improve its anti-acne effect. The incorporation of TC into vesicular systems, which provides effective drug delivery through skin, may open up a new market for pharmaceutical and cosmetic industries.

MATERIALS AND METHODS

Materials

Tetracycline hydrochloride was purchased from North China Pharmaceutical Goodstar Co (China). Cholesterol, rhodamine B, and methanol were obtained from Merck Chemicals Co. (Germany). Span 60, dimethyl formamide, ammonium oxalate, dibasic ammonium phosphate and ammonium hydroxide were purchased from Sigma-Aldrich (Germany). Soya lecithin was obtained from Lipoid Company (Germany). Chloroform was provided from Dae-Jung Chemicals Company (Korea). Ethanol was supplied from Golriz Company (Tehran, I.R. Iran).

Preparation of tetracycline-loaded nano-liposomes and nanotransfersomes

The vesicular nanostructures were prepared by thin film hydration method. Briefly, phosphatidylcholine and TC (6:1, w:w) were dissolved in chloroform and methanol, respectively and then the solution mixture was evaporated up to complete dryness (80 rpm, 40 °C, 110 mPa, and 30 min). A thin lipid film was hydrated with 10 mL phosphate buffer saline (PBS, pH 7.4, 10 mM) in a water bath at 60 °C for 1 h. Subsequently, vesicular nanostructures were sonicated using a 20 kHz ultrasonicator (UP200S, Dr. Hielscher; Germany) at 70% amplitude for 6 min. To prepare the formulation of transfersome, lecithin: span (4:1, w:w) was added to chloroform. rhodamine B labeled (dye content = 0.001%) liposomal and transfersomal nanostructures were also prepared to be used in *in vitro* skin penetration studies by imaging technique. The unloaded rhodamine B and TC were removed from the nanosuspensions by Amicon® tube as described later in drug loading calculation section and used for the *in vivo* and *ex vivo* studies.

Characterization of vesicular nanostructures

Particle size distribution of prepared nanostructures was analyzed by dynamic light scattering (DLS) method and reported as intensity-weighted average (z average) and the polydispersity index (PDI), which quantifies size and distribution width, respectively. Zeta potential of prepared liposomes and transfersomes were also analyzed by the same system (Nano ZS, Malvern, UK). Prior to measurement, samples were diluted with PBS. The morphology of the prepared

nanostructures was evaluated using scanning electron microscope (SEM) (MIRA3 TESCAN, UK) operating at 15 kV. The specimens were mounted on a metal stub with double-sided adhesive tape and coated under vacuum with gold (100-150 Å) in an argon atmosphere prior to observation using a direct current sputter technique (DST3, Nanostructured Coating Co., Tehran, I.R. Iran).

Determination of encapsulation efficiency and loading capacity

The encapsulation efficiency percent (EE%) and loading capacity percent (LC%) of TC incorporated into the nanovesicles were calculated using the following equations:

$$EE\ (\%) = \frac{W_{(encapsulated\ drug)}}{W_{(added\ drug)}} \times 100 \quad (1)$$

$$LC\ (\%) = \frac{W_{(encapsulated\ drug)}}{W_{(total\ lipid)}} \times 100 \quad (2)$$

where, $W_{(added\ drug)}$ is the amount of initial drug used for the preparation of nanoparticles, $W_{(encapsulated\ drug)}$ is the amount of drug loaded in the nanoliposomes, and $W_{(total\ lipid)}$ is the amount of lipid used in the preparation of liposomes or transfersomes. EE% and LC% were determined by first separation of the un-loaded drug by centrifugation method using of Amicon® Ultra-15 with the MWCO of 100 kDa (Millipore, Germany) tubes. The formulation sample was added to the upper chamber of the Amicon® tube and then the tube was centrifuged (Sigma 3K30, Germany) at 4000 rpm for 10 min (14). The clear solution in the bottom of Amicon® tube was used for the assessment of unloaded drug. The concentration of drug was determined by a validated high performance liquid chromatography (HPLC) method.

High performance liquid chromatography analysis of tetracycline hydrochloride

The samples' drug content were measured at room temperature using a reversed-phase HPLC method employing a C8 column (4.6 mm × 250 mm, 5µm) (Knauer, Germany). The diluent was dimethylformamide and ammonium oxalate (0.1 M) mixture (27:68). The mobile phase consisted of dimethylformamide, ammonium oxalate (0.1 M), and dibasic ammonium phosphate (0.2 M) (27:68:5). The flow rate, injection volume, detection wavelength, and the retention time were 1 mL/min, 20 µL, 274 nm, and 6.9 min, respectively. Calibration curve was linear in the range of 0.1-50 µg/mL and method was successfully validated and showed good linearity, reproducibility, and accuracy. The limit of detection (LOD) and limit of quantification (LOQ) were 30 ng/mL and 130 ng/mL, respectively. During the HPLC procedure, the samples were covered with aluminum foil to protect the drug from light and all samples were filtered through an aqueous 0.45 µm pore size filter membrane. No interference from the formulation components or skin tissue was observed.

In vitro drug release studies

The cells were setup on *in vitro* penetration study performed by Franz type diffusion apparatus (Erweka HDT6, Germany) equipped with 6 diffusion cells with diffusion area of 5.3 cm^2 using cellulose membrane (cutoff of 12 kDa, Sigma, St. Louis, MO, USA) with minimum three diffusion cells and repeated three times in different days. 25 mL PBS was used as the receptor medium and 500 µL of each prepared formulation was placed on the cellulose membrane in the donor chamber. Temperature of the receptor medium was maintained at 32 °C by circulating the warm water between two layers of diffusion cell and the receptor medium was magnetically stirred at 750 rpm. Samples of 400 µL were withdrawn from the receptor compartment at predetermined time intervals (30, 60, 120,180, 360, 720, 960, and 1440 min) and replaced with the same volume of buffer solution. The amount of TC in the receptor phase was measured with HPLC apparatus. The cumulative amounts of TC permeated through membrane were plotted as a function of time.

Ex vivo skin penetration study

Male Wistar rats (140-180 g; supplied from Pasteur Institute of Iran, Tehran, I.R. Iran) housed in animal facilities of Drug Applied Research Center of Tabriz Medical Science University, were used for percutaneous

absorption studies. Animals were fed with a regular rat chow diet and had free access to food and water under standard lighting and humidity conditions (12 h light: 12 h darkness). This study was conducted in accordance with the guidelines of the care and use of laboratory animals of Tabriz University of Medical Sciences, Tabriz, I.R. Iran (National Institutes of Health Publication No. 85-23, revised 1985). After sacrificing with excess chloroform inhalation, rat skin hairs of abdominal region were removed with an electric razor and rinsed with physiological normal saline solution. The abdominal skin was surgically excised and adhering subcutaneous fat was carefully cleaned. To remove extraneous debris and leachable enzymes, the dermal side of the skin was in contact with a saline solution for 12 h before starting the diffusion experiment. The skin was mounted between the donor and receptor chamber of Franz diffusion cell (the SC facing the donor compartment). The temperature of the receptor medium was maintained at 37 °C. Nanovesicular suspension (0.5 mL) and equivalent amount of TC solution were placed on the skin surface in the donor compartment. All Franz diffusion cells were covered with aluminum foil to prevent degradation of drug under the light exposure.

At the end of the experiments (12 h), the skins were removed and rinsed with distilled water to remove excess drug from the surface. The skin surface was washed with methanol and centrifuged for 10 min at 10000 rpm and then the supernatant was analyzed for drug content by HPLC method according to the reported method mentioned in above sections (repeated at least in triplicate). Furthermore, the amount of drug received to the receptor phase was analyzed with HPLC. Fluorescence measurement allows to investigate the distribution of dyes into skin and therefore enables to explore the skin deposition of carrier. Rhodamine B-loaded nanoparticles suspension (0.5 mL) were poured on the rat flank skin and calmly rubbed with latex glow covered finger on the rat skin. Nanoparticles contacted with rat skin for 3 h, then rats were scarified and the skin was cut by a freeze-microtome (Frigocut™ 2800 N, Leica, Bensheim, Germany) vertically in 10-µm slices. The dye distribution in the skin was investigated by fluorescence microscopic (BX50, Olympus, Japan) at 420 (visible), 510 (blue) and 580 (red) nm (15).

Anti-microbial assessment

The antimicrobial activity of the TC-loaded nanovesicles was evaluated against *S. epidemidis*. The microorganism was provided by the microbiological laboratory, Drug Applied Research Center, Tabriz University of Medical Science, Tabriz, I.R. Iran. A single colony from the stock was transferred into Mueller Hinton broth (MHB, Merck, Germany) and incubated overnight at 35 °C. After incubation time, the cells were harvested by centrifugation at 3000 rpm for 5 min, washed twice and re-suspended in saline solution to provide an optical density equal to 0.5 McFarland standard turbidity. The minimal inhibitory concentration (MIC) values were assessed using the microdilution method (11).

Two-fold serial dilutions of the TC-loaded formulations were prepared for MIC assessment of *S. epidermidis*. Prepared diluted solutions were transferred to the tubes and standardized microorganism suspensions (0.5 McFarland) were added and incubated at 35 °C for 24 h. After incubation time, turbidity of tubes was evaluated to determine bacterial growth and last dilution with no turbidity at wavelength of 620 nm (lack of growth) was considered as MIC. All procedures were performed under aseptic conditions and reproducibility was examined by triplicate examination. In each test, microorganism strain in MHB (with blank vesicular formulation) and MHB alone were used as a positive and negative growth controls, respectively. To confirm the results three separate experiments were performed (16).

Data analysis

Statistical analysis was performed by a one-way analysis of variance (ANOVA) using a Tukey honest significant difference (HSD) test with SPSS software (version 16.0, Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

RESULTS

Characterization of tetracycline-loaded liposomal and transfersomal nanovesicles

The particle size and distribution of prepared TC-loaded liposomal and transfersomal formulations are represented in Fig. 1a and 1b which were found to be 74.8 ± 9.5 nm and 78 ± 11.5 nm as well as 0.26 ± 0.03 and 0.28 ± 0.02, respectively. The zeta potential values of liposomal and transfersomal nanovesicles were 17.2 ± 5.2 and 7.53 ± 3.84 mV (Fig. 1c and 1d), respectively. SEM image of optimum liposomal and transfersomal formulations are presented in Fig. 1e and 1f which verified the reported size characteristics (sub 100 nm size with uniform distribution) of nanovesicles. Optimum liposomal formulation showed the EE% and LC% of 44.9 ± 2.9 % and 8.39 ± 1.3 %, respectively. Corresponding values for transfersomes were found to be 54.6 ± 13.3 % and 10.92 ± 2.67 %, respectively.

In vitro drug release study

As shown in Fig. 2, the release profiles of vesicular formulations showed that percentage of released TC from liposomal formulation (55 ± 5.5 %) was higher than that of transfersomal formulation (21.6 ± 4.6 %) indicating maintaining the drug entrapped until its delivery to the target tissue and microorganism and preventing drug leakage.

Ex vivo drug permeation and deposition studies

The cumulative percentage of permeated TC from the optimized vesicular formulations and aqueous solution through excised rat skin was investigated using Franz diffusion cell for a period of 24 h (Fig. 3). As shown, the ratio of drug passed through the SC was increased by the encapsulation of TC into nanotransfersomes and nanoliposomes 3.2 and 1.9 folds, respectively higher than TC aqueous solution (Fig. 3a). In spite of higher skin penetration of nanotransfersomal TC than liposomal one (Fig. 3a), lower amounts of TC was appeared in receptor phase of Franz diffusion cell from nanotrasfesomes than nanoliposomes (Fig. 3b) indicating the less possibility of systemic absorption and consequently less side effect of nanostransfersomes.

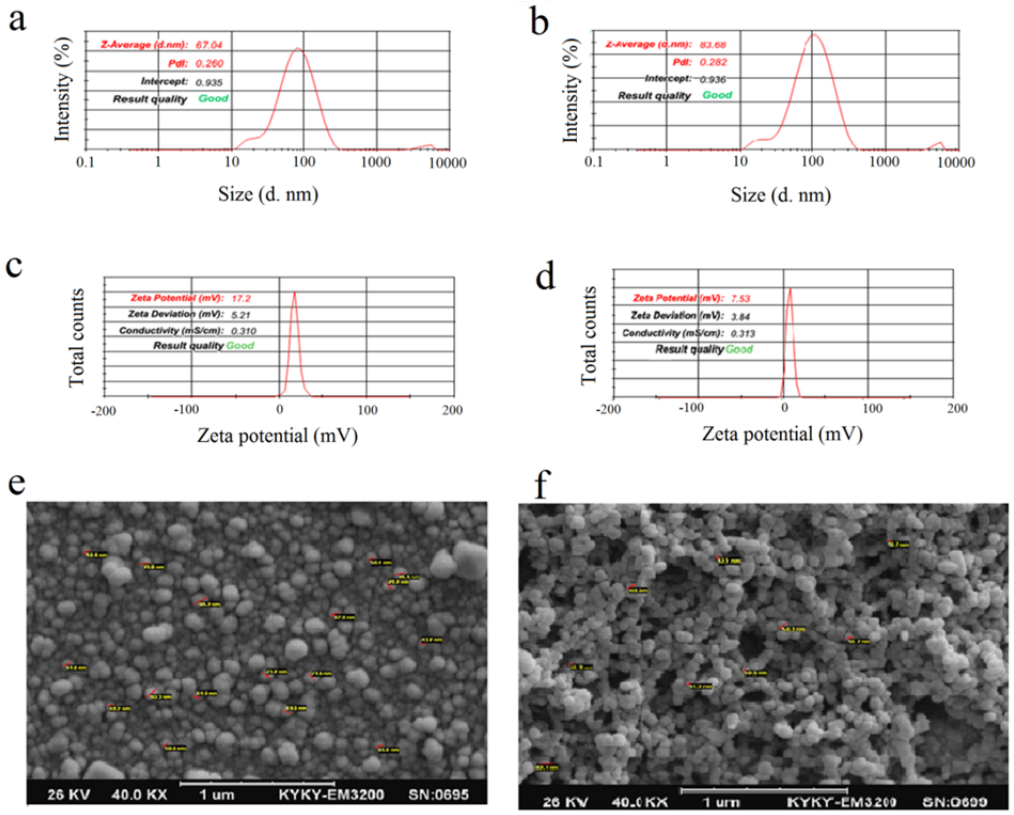

Fig. 1. (a, b) Particle size, (c, d) Zeta potential, and (e, f), scanning electron microscopy image of the optimized tetracycline-loaded liposomes (left) and transfersomes (right).

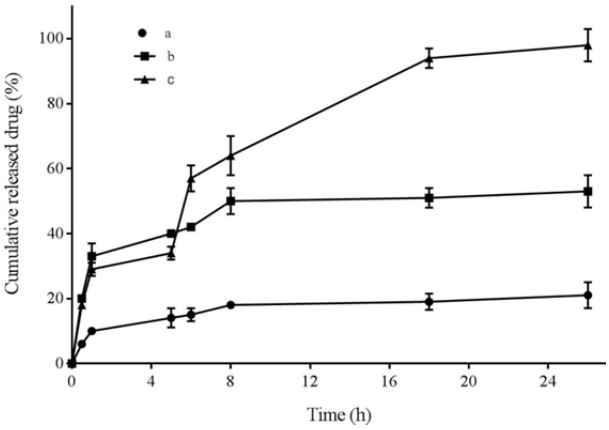

Fig. 2. *In vitro* drug release of tetracycline from transfersomal (a), liposomal (b), and aqueous solution (c) formulations.

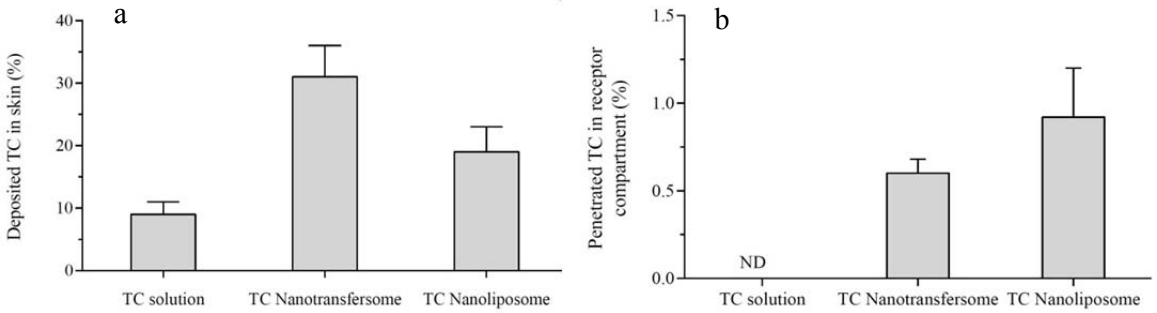

Fig. 3. Tetracycline (TC) (a) deposited in the skin and (b) permeated in to the receiver compartment of the Franz cell from aqueous, nanoliposomal, and nanotransfersomal formulations. ND, not determined.

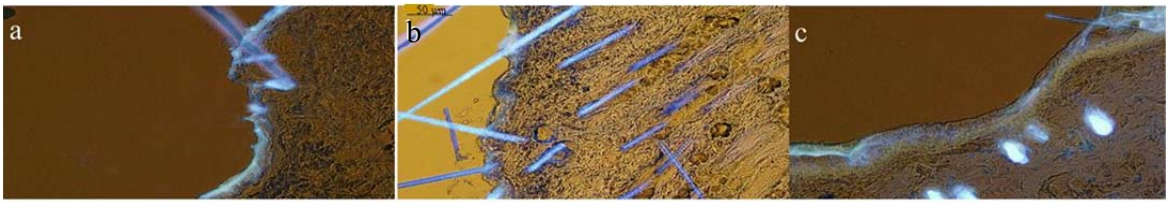

Fig. 4. Skin penetration capability of (a) rhodamine B solution, as well as (b) rhodamine B-loaded nanoliposomes, and (c) nanotransfersomes after 3 h of administration on skin.

Figure 4 shows the skin penetration capability of rhodamine B solution (Fig.4a) as well as rhodamine B-loaded nanoliposomes (Fig. 4b) and nanotransfersomes (Fig. 4c) after 3 h of application on skin.

The results illustrated that although the physicochemical characteristics of rhodamine B (MW of 422 and Log P of 1.95) are suitable for skin penetration (MW < 500 and Log P 1-4) (17,18), no evidence of skin penetration of rhodamine B was observed even after 3 h.

Antimicrobial efficacy of tetracycline solution, nanoliposomes and nano transfersomes

Antimicrobial test results showed that although liposomal and transfersomal formulations showed suitable size (sub 100 nm) and appropriate drug loading, the MIC values of TC solution (89.2 µg/mL, Fig. 5a) against *S. epidermidis* standard strains did not differ with those observed for the TC-loaded nanoliposomes (Fig. 5b) and nanotransfersomes (Fig. 5c).

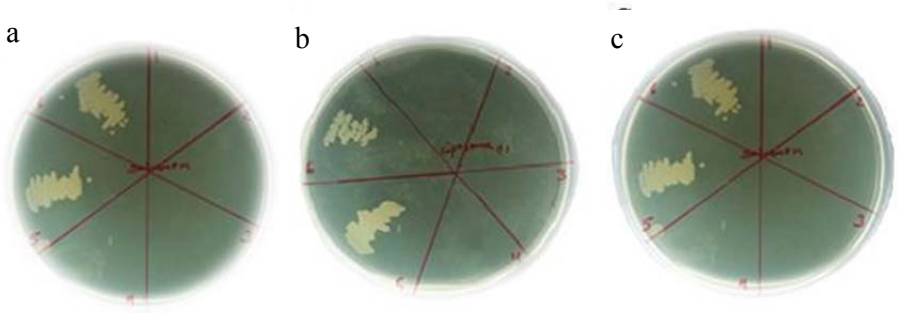

Fig. 5. The antibacterial effect of (a) tetracycline aqueous solution, (b) tetracycline-loaded nanoliposomes, and (c) nanotransfersomes against *Staphylococcus epidermis* standard strains**.**

DISCUSSION

The low PDI value (≤ 0.3) indicated the narrow size distribution of prepared formulations (19). The zeta potential values of both liposomes and transfersomes were less than ± 30 indicating lack of colloidal stability of suspended nanovesicles (20). Hopefully, topical aqueous gel can be suggested for the formulation of final dosage form of vesicular nanostructures (21) to guarantee the colloidal dispersion stability by entrapment of nanostructures in the polymeric lattice. Due to the high hydrophilicity of TC, these high EE% and LC% of both formulations was which might be due to the positive charge of entrapped active ingredient. Lecithin composes of negatively charged phosphate and positively charged choline groups which provided a totally negative charge (-26 mV, data was not shown) for the blank nanoliposomes. TC is a positively charged molecule and probably in addition to the physical entrapment of hydrophilic drug into the inner aqueous space of nanovesicles, ionic interaction of TC and lecithin in the inner and outer surface of vesicles played a positive role in the high reported LC% values for drug. This may be the reason of increasing of vesicles zeta potential value from negative value to positive. The flexibility in bilayer structure of transfersome and its lesser rigidity than liposomes might be responsible for lower drug release from transfersomal formulation (22). The lack of drug leakage before reaching to the target would be more important in nanoparticles due to their high surface to volume ratio which makes them much more potential for unwanted drug release. Providing flexibility in the surrounded wall of nanoparticles seems to be an appropriate strategy to overcome this obstacle (23). TC in the aqueous solution permeated lower than the vesicular nanostructures that might be attributed to the characteristics of TC (molecular weight (MW) = 480.90 g/mol and Log P = 0.62) which are not suitable for skin penetration (MW < 500 and Log P 1-4) (17,18). In spite of higher (1.6 fold) skin penetration of TC mediated by nanotransfersomes than nanoliposomes, lesser amounts (0.45 folds) of TC were detected in the receiver compartment of the Franz cell in the nanotransfersomes than nanoliposomes. This would be ideal for the delivery of drugs such as antibiotics, especially for those which may cause adverse systemic side effects, such as TC. Both higher skin penetration and lower drug entrance to the receiver compartment concluded the superiority of nanotransfersomes in comparison with nanoliposomes in dermal delivery of TC. Thus, drug-localizing effect in the skin seems possible with nanotransfersomal formulation enabling drug targeting to the skin and reducing toxic side effects of drug by decreasing systemic absorption which responses to an interesting topic in pharmaceutics due to the problems in dermal directing a ionized and hydrophilic drug e.g. TC (24,25). A minor penetration of rhodamine B solution might be due to close contact with superficial junctions of the SC and the furrows between the corneocytes islands and hair follicles, allowing superficial spreading of the drug. MW of rhodamine B is near to 500 and this may be a reason. Probably, MW and Log P are not only the determining parameters

for skin penetration. Rhodamine B is a positively charged molecule (such as tetracyclic) and this might be an another reason for lacking topical penetration through skin. It was recently reported that charged materials (negative or positive) show less skin penetration (26). However, the deep penetration of rhodamine B-loaded vesicles compared to unpenetrated rhodamine B solution state proved the key role of vesicles in efficient dermal drug delivery. The accumulation of liposomes limited only in the hair follicles and sweat glands, while transfersomes penetrated deeply in skin layers. Results also indicated that nanotransfersomes could transfer rhodamine B into the deeper layer of skin. Therefore, it can be concluded that transfersomes are better carrier for dermal delivery of TC and anti-acne treatment. The general hypothesis was that nanoparticles improve the penetration of antimicrobial agents into bacteria and increase the efficiency and decrease the MIC value (27). On the other hand, a few reports resulted in the inefficiency of nanoparticles against microbial strains (28-31). It might be concluded that nanoparticles would be an ideal carrier for drug facing difficulties in entering into bacteria and probably TC is not the case. Our results at least indicated that TC did not go under any interaction, chemical or physical incompatibility during entrapment into the vesicular nanostructures and drug acted the same as free drug. Although the nanovesicular carriers did not improve the anti-microbial activity of TC, their advantage in superior dermal delivery probably results in better clinical outcomes.

CONCLUSIONS

The results reported in the present study indicated that the composition of vesicles bilayer improved the dermal delivery of TC. Compared with aqueous solution, vesicular nanostructures, especially transfersomes, showed better accumulation of TC in skin and revealed a significant skin targeting effect, which might result from the special characteristics of these nanoparticles. In conclusion, the current study suggested that nanotransfersomal formulations have a great potential for topical drug targeting and may be used as a feasible cargo carrier for the cutaneous delivery of various drugs.

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