https://doi.org/10.31925/farmacia.2022.3.18 **ORIGINAL ARTICLE**

IMPROVED SKIN PENETRATION AND DEPOSITION OF NAFTIFINE FROM TRANSETHOSOMES AND TRANSETHOSOMAL GEL FORMULATIONS

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Abstract

The main objective of the present study was to develop and characterize naftifine loaded transethosomes and their comparison with marketed cream of naftifine (Exoderil®, Sandoz Türkiye) for enhanced dermal delivery. Transethosomes loaded with naftifine were developed using the film dispersion method and evaluated for particle size and size distribution, zeta potential, drug recovery yield and stability. The optimal transethosome formulation with a mean particle size of 50.20 ± 0.22 nm, polydispersity index of 0.056 ± 0.024 and drug recovery of 96.215 ± 3.341% was incorporated into hydroxypropyl cellulose, hydroxypropylmethyl cellulose and chitosan gels. The *in vitro* pig skin penetration of naftifine from transethosome dispersions and transethosomal gel formulations was determined quantitatively by using sequential tape stripping method and compared with the commercial product. The ability of Nile red loaded transethosomes to reach the lower layers of skin was visualized by confocal laser scanning microscopy (CLSM) and ATR-FTIR spectroscopy has been used to study the interaction of transethosomes with the *stratum corneum* lipids. In conclusion, a topical transethosomal system could be a potential carrier to overcome the *stratum corneum* barrier and enhance skin penetration and deposition of naftifine.

Rezumat

Obiectivul principal al prezentului studiu a fost acela de a dezvolta și caracteriza transetozomii încărcați cu naftifină și compararea acestora cu crema de naftifină comercializată (Exoderil®, Sandoz, Turcia) pentru o cedare cutanată îmbunătățită. Transetozomii încărcați cu naftifină au fost dezvoltați prin metoda de dispersie a filmului și evaluați pentru dimensiunea și distribuția mărimii particulelor, potențialul zeta, recuperare și stabilitate. Formularea optimă de transetozom cu o dimensiune medie a particulei de 50,20 ± 0,22 nm, indice de polidispersitate de 0,056 ± 0,024 și o recuperare a naftifinei de 96,215 ± 3,341% a fost încorporată în geluri cu bază de hidroxipropil celuloză, hidroxipropilmetilceluloză și chitosan. Penetrarea prin piele de porc a naftifinei din dispersii transetozomale și formulări de gel transetozomal a fost determinată cantitativ prin utilizarea metodei de stripare secvențială și comparată cu produsul comercial. Capacitatea transetozomilor încărcați cu roșu Nil de a ajunge în straturile inferioare ale pielii a fost vizualizată prin CLSM și spectroscopia ATR-FTIR a fost utilizată pentru a studia interacțiunea transetozomilor cu lipidele stratului cornos. În concluzie, un sistem transetozomal topic ar putea fi un purtător potențial pentru a depăși bariera stratului cornos și pentru a îmbunătăți penetrarea prin piele și sedimentarea naftifinei.

Keywords: transethosomes, naftifine, topical drug delivery, colloidal drug delivery systems

Introduction

Many different approaches are being studied to improve the penetration of drugs into the skin. The use of chemical penetration enhancers or physical methods (iontophoresis, sonophoresis and electroporation) and the development of specific drug delivery systems are the main strategies used [20, 23]. The development of vesicular carriers such as liposomes, transferosomes and ethosomes has led to a significant improvement in the dermal application of drugs. Transethosomes, among these systems, have attracted a lot of attention in recent years. They are vesicular systems whose composition is based on phospholipids, ethanol, water and surfactants. Transethosomes encompass the advantages of transferosomes and ethosomes: higher skin penetration and higher deformability compared to conventional liposomes [25, 38]. They were first presented by Song *et al*. [32], and it was shown that the presence of a surfactant together with ethanol in the vesicle structure constitutes a system with better physicochemical characteristics than conventional vesicular carriers [7]. Thanks to their remarkable dermal and transdermal potential, transethosomes are being studied for the administration of various drugs for the treatment of various cutaneous pathologies. Recent studies have been focused on loading antioxidants (i.e., mangiferin, coenzyme Q10, resveratrol, (-)-epigallocatechin-3-gallate, fisetin) [3, 8, 9, 27, 31], antifungal drugs (i.e., econazole nitrate) [37], hormones (i.e., progesterone) [30], antihypertension agents (i.e.,

olmesartan medoxomil) [1] or other compounds such as imiquimod, rosmarinic acid, vitamin E and caffeine to transethosomes in order to investigate their dermal or transdermal effectiveness [3, 4, 25, 28].

The leading aetiology of superficial and cutaneous fungal infections is caused by dermatophytes of the *Microsporum*, *Trichophyton* and *Epidermophyton* genera, and approximately 20 - 25% of the world's population is affected by these infections [21, 38]. In the treatment of fungal infections of the skin, topical treatment is generally preferred to systemic therapy. The topical efficacy of antifungal drugs could be limited due to inconvenient physicochemical characteristics and side effects. Vesicular carriers can be very effective for treating fungal skin infections due to their ability to alter the penetration of active agents to deeper skin layers and target the diseased region of the skin [38]. Naftifine hydrochloride (naftifine) is a broad-spectrum allylamine derivative antifungal drug, most effective against superficial dermatophytes. The molecular mechanism of the drug is the inhibition of the enzyme squalene epoxidase at an early stage in fungal ergosterol biosynthesis [12]. There are conventional preparations of naftifine in the form of cream, gel, and solution in the international market. Because of its high lipophilicity (logP: 5.4), naftifine tends to accumulate in the *stratum corneum*, the outermost layer of the skin, thus restricting its penetration into the deeper layers in the topical treatment of fungal infections [10, 11, 39]. Thus far, a few studies have been reported on the development of cyclodextrin [35, 36], β-cyclodextrin hydrogel [26], niosome gel [5] and microemulsion [10] type carriers of naftifine. To our knowledge, transethosomal nano-carriers of naftifine have not been reported in the research literature.

In our study, a novel transethosomal nanocarrier was prepared for enhanced dermal delivery of naftifine. The physicochemical characteristics of the optimized transethosome formulation, including particle size, size distribution, zeta potential, and drug recovery yield, were determined, and its stability was investigated. *In vitro* skin penetration and deposition behaviour of naftifine transethosomes and three gel preparations containing naftifine transethosomes were assessed by sequential tape stripping, confocal laser scanning microscopy (CLSM) and Attenuated Total Reflectance Fourier Transformed Infrared (ATR-FTIR) spectroscopy in comparison with a marketed cream of naftifine (Exoderil®, Sandoz Turkey).

Materials and Methods

Materials

Naftifine was kindly provided from Zentiva (Istanbul, Turkey). Phospholipon 90G (Purified phosphatidyl-choline from soybean lecithin, P90G) was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Poly-oxyethylene sorbitan monooleate (Tween 80), chitosan (high molecular weight), and Nile red were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Hydroxypropyl cellulose (HPC, Klucel™ Grade H) and hydroxypropyl methylcellulose (HPMC, Type 60 M) were supplied from Asland Inc. (Lexington, USA) and Daido Chemical Corporation (Tokyo, Japan). All other reagents used were analytical grade.

Preparation of naftifine loaded transethosome formulation

Transethosomes loaded with naftifine were prepared by the thin-film hydration method [32]. Briefly, phospholipid, drug and surfactant were dissolved in organic solvent with a ratio of 2:1 (chloroform:methanol) in a pear-shaped rotary evaporator flask. This mixture was evaporated under vacuum (Buchi Rotavapor R-210, Switzerland) at 40°C until a thin lipid film was formed on the wall of the flask. The dry lipid film was hydrated with 30% (v/v) ethanol solution by rotation at 100 rpm for 1 h at room temperature. The formed vesicles were allowed to swell for 2 h at room temperature, and sonication was performed three times at five-minute intervals with a probe sonicator (Sonics VibraCell, 20 kHz, 50% energy output) to both reduce the size of the vesicles and narrow the size distribution. For further use, the transethosomal dispersion was tightly sealed and preserved at 5 ± 3°C [32]. The composition of the optimum transethosome formulation (TE) obtained as described above is represented in Table I.

Table I

Composition of the transethosomal formulation (TE) containing the naftifine

Ingredient	% (w/v)
Naftifine	0.3
Phospholipid	4
Tween 80	0.5
Ethanol	30

Determination of particle size, polydispersity index, and zeta potential

The mean particle size, polydispersity index (PI) and zeta-potential of freshly prepared transethosome dispersion were measured by dynamic light scattering using the Malvern NanoZS (Malvern Instruments Ltd., UK) at room temperature.

Determination of drug recovery yield

The recovered naftifine amount in the transethosomes was quantified by HPLC (Shimadzu, Japan) after removing the undissolved drug by a membrane filter (0.2 µm, Minisart, Sartorius stedim, Germany). The vesicles were disrupted with 20% Triton X-100 aqueous solution. The recovery yield of the drug in the transethosomal formulation was calculated as

$$\text{Recovery yield (\%)} = (Ft/Fi) \times 100, \quad (1)$$

where, Ft is the total amount of naftifine in the transethosome preparation and Fi is the initially added amount of the drug [32].

Stability of naftifine transethosomes

To examine the physical stability of the naftifine loaded transethosomes that were kept at 5 ± 3°C, the mean particle size and the polydispersity index at the end of the first- and third months following their preparation were measured with a particle size analyser (Malvern Instruments, Malvern, UK). Also, the drug recovery yield of the optimized formulation at the end of the first- and third months was determined by HPLC (Shimadzu, Japan).

Incorporation of naftifine transethosomes in hydrogels

Naftifine transethosomes (TE) were incorporated into HPC (2%, w/v), HPMC (2%, w/v) or CHIT (2%, w/v) gels, respectively, with the aim to enhance topical application to skin. Briefly, the polymer (HPC or HPMC) was dissolved in water under continuous stirring at 800 rpm, and the formed gel was stored in airtight glass jars at room temperature for 24 hours to allow the complete swelling of the polymer. Chitosan gel was prepared by dissolving 2% (w/v) of high molecular weight chitosan in 1% (v/v) aqueous acetic acid under constant stirring. To these gels, naftifine transethosomes (2 mL) were added and mixed to form a uniform dispersion of transethosomal gel.

In vitro studies

Skin penetration assessment by sequential tape stripping

The *in vitro* skin penetration and deposition studies of naftifine transethosomes (TE) and naftifine transethosomal gels (TE-HPC, TE-HPMC and TE-CHI) compared to commercial Exoderil® 1% Cream were conducted on total thickness dorsal pig skin. The pig skin was obtained from a local slaughterhouse and stored at -20°C till further use. The penetration study was performed using Franz diffusion cells with a receptor compartment capacity of 12 mL and an effective diffusion area of 1.77 cm^2. Skin samples (n = 6 *per* formulation) from which the subcutaneous fat layer has been removed were clamped between the donor and the receptor chamber of Franz diffusion cells, with the *stratum corneum* side facing the donor compartment. The receptor compartment was filled with PBS solution (pH 7.4), which was continuously stirred using a magnetic bar at 250 rpm. The temperature was kept at 37 ± 0.5ºC. An amount equivalent to 6 mg drug of each formulation (TE, TE-HPC, TE-HPMC, TE-CHI and commercial cream) was placed on the donor compartment. At the end of the *in vitro* skin penetration study (24 h), 1 mL of receptor phase was sampled, and then the skin specimens were collected from diffusion cells. The skin surfaces were washed several times with fresh receptor fluid before stretching the skin on styrofoam plates and fixing it with needles. The cutaneous penetration and deposition of naftifine were investigated using sequential tape stripping procedure as described in our previous publications [10, 13]. Briefly, twenty consecutive tape strips (Scotch® Book Tape 845, 3M, USA) were obtained from the diffusion area of each skin. All strips were immersed in 3 mL acetonitrile:tetrahydrofuran:tetramethyl-ammonium hydroxide buffer (62:10:28, pH 7.8), followed by shaking on an orbital shaker (Thermo Forma Orbital Shaker, USA) for 12 hours at ambient temperature. After removing the pieces of tape, the extracted samples were filtered through 0.45 µm Millex LH filters and directly analysed using HPLC. To determine the amount of naftifine in the skin remaining from the tape stripping, the skin was cut into small pieces with surgical scissors and extracted with the same procedure.

ATR-FTIR spectroscopy

At the end of the *in vitro* penetration study, skin contacted with naftifine transethosomes (TE) and transethosomal gel formulations (TE-HPC, TE-HPMC and TE-CHI) was investigated using ATR-FTIR (Perkin Elmer Spectrum 100 FT-IR Spectrometer, Shelton, CT, USA) to obtain the molecular functional group vibrations of the lipids in the *stratum corneum*. Spectra were collected by placing the sample with the *stratum corneum* facing the ATR crystal. In order to minimize the variation between the samples, the same skin piece was used for normalization before treatment and normalization procedure was performed according to the literature [15, 17]. Perkin Elmer Spectrum Version 6.0.2 software was used to determine the peak positions.

Skin penetration study with Nile red labelled transethosomes

To visualize the penetration of transethosome formulation TE into different skin layers, the vesicles labelled with 1.25×10^{-3}% (w/w) Nile red (log P: 5.0) were applied to pig skin for 24 h under the same *in vitro* skin penetration study conditions [22]. Then, the skin samples were frozen, and the distribution of the fluorescent marker in skin layers was detected using Leica TCS SPE CLSM (Leica, Wetzlar, Germany) with 20× magnifications (209 objective lens, Zeiss Micro- systems, Germany). A HeNe diode laser was used to excite Nile red at 543 nm with emission fluorescence at 630 nm that did not interfere with the background fluorescence caused by skin protein bands [15]. A suspension of Nile red (1.25×10^{-3}%, w/w) in PBS (pH: 7.4) served as the control.

HPLC analysis

The quantitative determination of naftifine in this study was performed using a Shimadzu HPLC system (Shimadzu Model LC 20AT; Shimadzu Corporation, Kyoto, Japan). A reversed phase C18 column (4.6 x 150 mm, 5 µm; EMD Millipore, Billerica, MA, USA) preceded by a guard column (44 mm, 5 µm, Merck) was used as a stationary phase. The mobile phase consisted of acetonitrile:tetrahydrofuran:tetramethyl-ammonium hydroxide buffer (62:10:28, pH 7.8). The flow rate and detection wavelength were set at 1.2 mL/min and 280 nm, respectively. The temperature was adjusted to 30°C. The HPLC method was validated for selectivity, linearity, accuracy and precision. It was found to be linear between the concentration range 0.025 - 100 µg/mL with a high correlation coefficient

($r^2 > 0.999$) and precise (intra- and inter-day variation < 2%) and accurate (mean recovery > 99%). The extracted pig skin and blank tapes did not show any chromatogram peaks interfering with naftifine, confirming the method's selectivity.

Statistical analysis

All results are the mean ± standard deviation of at least three to six experiments. The statistical analysis was performed using the one-way ANOVA, Tukey's Multiple Comparison Test, with $p < 0.05$ as the significance level (GraphPad Software, Inc., Version 5, La Jolla, CA, USA).

Results and Discussion

Determination of particle size, polydispersity index, and zeta potential

Based on pre-formulation studies, the optimum transethosome formulation providing a homogeneous and reproducible vesicle distribution in nanometre size was prepared by using 4% (w/v) phospholipid, 0.5% (w/v) Tween 80 and 30% (w/v) ethanol were dissolved in chloroform and methanol in the ratio of 2:1. The concentration of naftifine loaded into the vesicles was 0.3% (w/v). The transethosome formulation TE was uniform and almost transparent liquid with a mean particle size of 50.20 ± 0.22 nm, and the PDI value was 0.056 ± 0.024 (Table II). The ultrasonication process enabled vesicles to be obtained more homogeneously, with smaller particle size and narrower distribution. The zeta potential of the transethosomes was -10.9 ± 0.7 mV.

Determination of drug recovery yield

The average drug recovery yield was 96.215 ± 3.341% (Table II). It was assumed that the high recovery of naftifine in the vesicles might be due to the drug solubilization effect of ethanol contained in the vesicles.

Table II

Physicochemical characteristics of optimized transethosome formulation TE

Particle size (nm)	50.20 ± 0.22
Polydispersity index (PdI)	0.056 ± 0.024
Zeta potential (mV)	-10.9 ± 0.7
Drug recovery yield	96.215 ± 3.341 %.

Stability of naftifine transethosomes

Figure 1 presents the particle size and the drug recovery yield of transethosomes that were kept at 5 ± 3°C and measured at first month and third months following vesicle preparation. The analytical results indicate that the mean particle size and the recovery yield of transethosomes did not change significantly ($p > 0.05$) over time. This result is in accordance with the finding that the transethosomes can prevent drug leakage due to the surfactants (edge activators) in their formulation [33].

Incorporation of naftifine transethosomes in hydrogels

Naftifine loaded transethosome formulation TE was incorporated into the gel base of different polymers to improve applicability by increasing the skin residence time of the formulation. Homogenous gels were obtained using 2% (w/v) HPC, 2% (w/v) HPMC or 2% (w/v) CHIT. When the prepared gel formulations were visually inspected, HPC and HPMC gels were white while CHIT gel was yellowish white. Indeed, all the gel formulations were homogenous, translucent, and applicable to the skin efficiently.

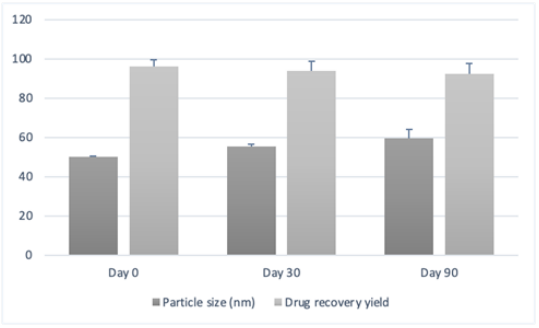

Figure 1.

The particle size and the drug recovery yield of naftifine transethosomes at first- and third months following vesicle preparation

In vitro studies

Skin penetration assessment by sequential tape stripping

In an effective topical antifungal therapy, the drugs should cross the *stratum corneum*, which is the main barrier to the cutaneous penetration of substances, and reach the deeper layers of the skin, especially the viable epidermis [11, 32].

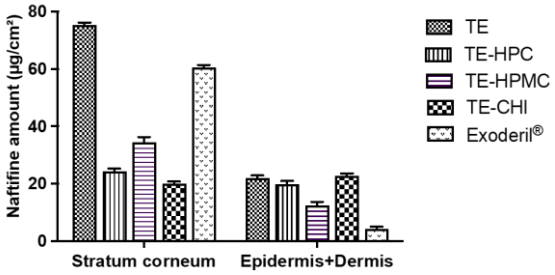

Figure 2.

The amounts of naftifine accumulated into the *stratum corneum* and the rest of the skin (epidermis + dermis) after removing *stratum corneum* by tape stripping for the transethosomes, transethosomal gels and Exoderil® 1% Cream as control

Figure 2 shows the profiles of naftifine localized in the *stratum corneum* and residual skin (epidermis + dermis) 24 h after the application of the formulations to pig skin. Transethosomal gel formulation TE-CHI showed the highest naftifine accumulation in the epidermis + dermis (22.681 ± 0.840 µg/cm^2), followed by the transethosome dispersion TE (22.060 ± 0.740 µg/cm^2), the gel formulations TE-HPC (19.851 ± 1.030 µg/cm^2) and TE-HPMC (12.519 ± 1.121 µg/cm^2) and the commercial cream Exoderil® (4.138 ± 0.852 µg/cm^2).

The significantly higher ($p < 0.05$) drug deposition into the deeper layers seemed to represent that the transethosomes and transethosomal gels were able to overcome the *stratum corneum* barrier and provided a considerable amount of naftifine accumulation in the skin.

Our results indicated that the conventional cream formulation acted as a reservoir only in the upper layer of skin (60.557 ± 0.825 µg/cm^2) and showed limited penetration in depth after 24 h study. Although transethosome dispersion TE provided the highest accumulation of naftifine in the *stratum corneum* (75.441 ± 0.700 µg/cm^2), drug localization in the lower layers of the skin was found to be significantly higher ($p < 0.05$) than in the conventional cream. It is considered that the nano-size of the vesicles and the ethanol in their composition might be effective in improving the penetration of naftifine into the skin [1, 4], whereas the conventional form is confined to the *stratum corneum* as compared to transethosomes and transethosomal gels.

ATR-FTIR spectroscopy

ATR-FTIR spectroscopy is a valuable technique used to investigate the molecular effects of different formulations on the conformational order of skin lipids and proteins. The bands of asymmetric and symmetric C-H stretching modes (2920 cm^{-1} and 2850 cm^{-1}, respectively) represent the vibrations of lipids present in *stratum corneum* [6, 14, 18]. Figure 3 shows the effect of the naftifine transethosomes (TE), transethosomal gels (TE-HPC, TE-HPMC and TE-CHI) and Exoderil® on the frequencies of the C–H symmetric and asymmetric absorbances. Compared to untreated control skin, the C-H stretching vibrations were detected at noticeably higher wavenumbers in the skin samples treated with TE and TE-CHI, indicating a higher barrier permeability. The asymmetric stretching vibrations shifted from 2918.27 ± 0.01 cm^{-1} (untreated control skin) to 2919.65 ± 0.01 cm^{-1} and $2919{,}50 \pm 0.02$ cm^{-1} for the transethosome formulation TE and transethosomal gel formulation prepared with chitosan polymer (TE-CHI), respectively. The symmetric stretching vibrations were obtained at 2850.54 ± 0.02 cm^{-1} for control skin, whereas TE and TE-CHI induced blueshift on the peak position of the C-H symmetric stretching vibration ($\nu_s = 2851.29 \pm 0.01$ cm^{-1} and $\nu_s = 2851.12 \pm 0.01$ cm^1, respectively) indicating that the *stratum corneum* intercellular lipids were disordered. This phenomenon did not occur in the samples treated with TE-HPC, TE-HPMC and Exoderil®. These results are in agreement with data from the *in vitro* skin penetration assessment study. It has been suggested that ethanol in the transethosome formulation interacts with *stratum corneum* intercellular lipids, causing fluidization of lipid multiple layers and thereby increasing mobility [7]. It is also reported that chitosan improves the penetration of active compounds by interacting with epidermal tight junction proteins that form a barrier in the stratum granulosum, below the lipid barrier in the [24, 34].

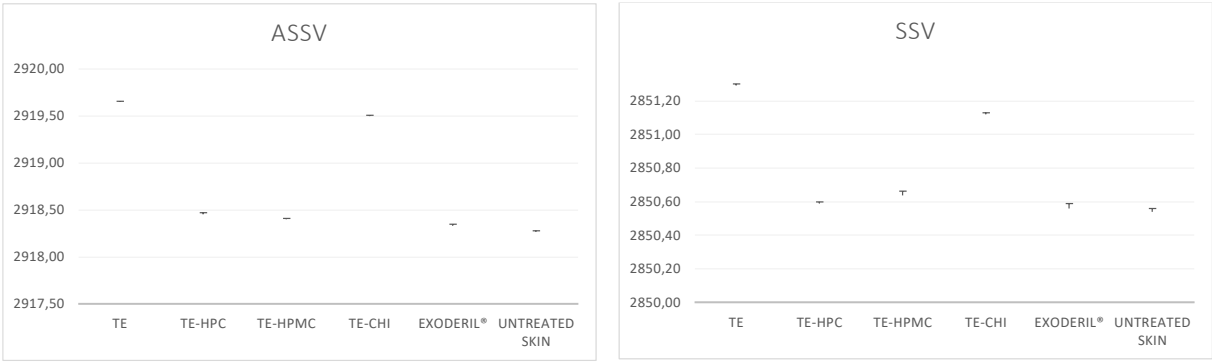

Figure 3.

Peak shifts of skin lipids in C–H asymmetric and symmetric stretching absorbances due to the application of the naftifine transethosomes TE, transethosomal gel formulations TE-HPC, TE-HPMC and TE-CHI and the conventional cream (Exoderil®) (n = 3)

Skin penetration study with Nile red labelled transethosomes

CLSM is an effective imaging technique used to visualize the depth of penetration and the possible penetration pathways through the skin [2, 16, 29]. Nile red, a fluorescent lipophilic dye, was loaded into transethosome formulation TE to mimic the lipophilic drug naftifine [22]. Also, a Nile red suspension in PBS (0.05%, w/w) was used as control.

The CLSM images of horizontal sections of pig skin (Figure 4) reinforced that Nile red loaded transethosomes could reach the fluorescent dye up to the deeper layers of skin so that there were high fluorescence intensities at the depths of 20 µm (Figure 4A1, representing the *stratum corneum* layer), 140 µm (Figure 4A2, representing the epidermis layer) and 500 µm (Figure 4A3, representing the dermis layer) from the skin surface after 24 hours. Hence, it can be concluded that the prepared transethosomes facilitated

Nile red cross the *stratum corneum* barrier and effectively delivered it into the deeper layers of the skin, which is desirable for the topical treatment of invasive fungal infections of the skin.

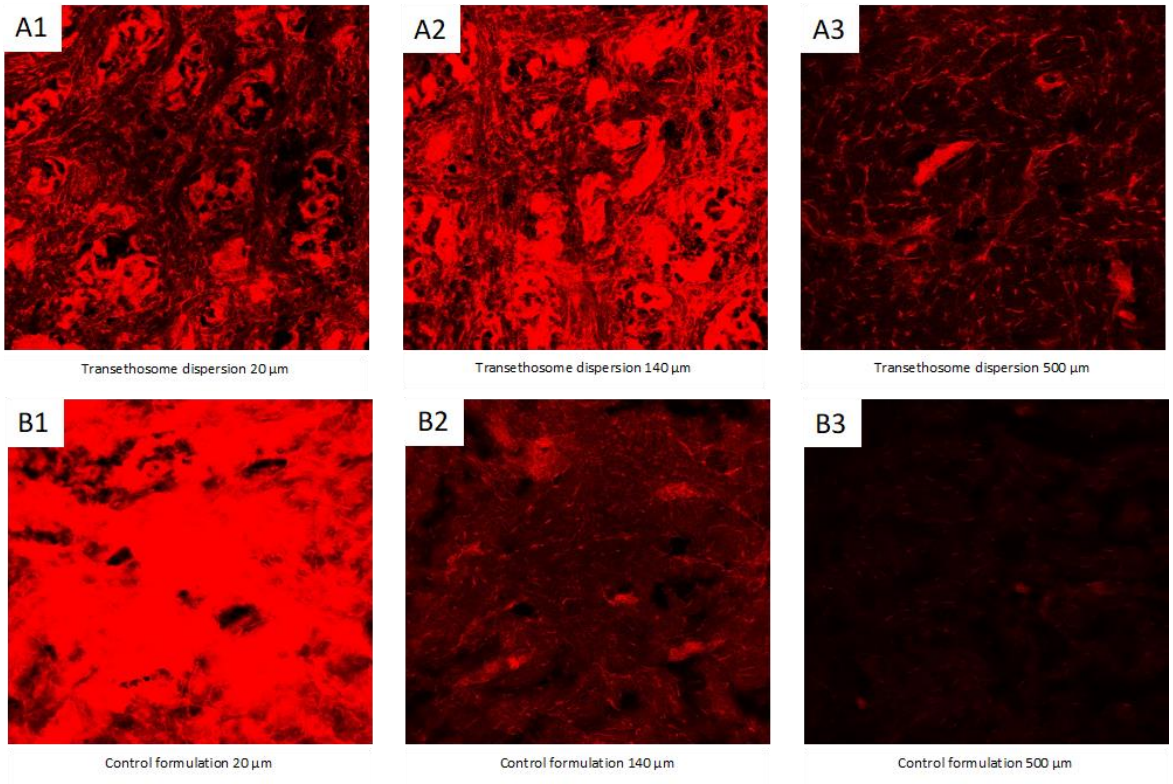

Figure 4.
CLSM images of horizontal sections of pig skin treated with A) Nile red loaded transethosomes and B) Nile red suspension in PBS as control at the depths of 20 µm (A1 and B1), 140 µm (A2 and B2) and 500 µm (A3 and B3) from the skin surface

Conclusions

In fungal skin infections, topical therapy is generally preferred to oral therapy. However, the most important obstacle to be overcome in the treatment with topical antifungal preparations applied in the form of conventional creams, gels or ointments is the barrier formed by the *stratum corneum*, the outermost layer of the skin, against permeability. The physicochemical properties of the active substance also come to the fore in penetration through the skin.

In this study, a transethosomal carrier of a highly lipophilic antifungal agent, naftifine, was developed and evaluated for its penetration and deposition in skin. After performing the physicochemical characterization studies, the optimized transethosome formulation (TE) was incorporated into HPC (2%, w/v), HPMC (2%, w/v) or CHIT (2%, w/v) gels, respectively, with the aim to enhance the topical application to the skin. The skin penetration of naftifine from transethosomes and transethosomal gels was studied *in vitro* using pig skin. Compared with the commercial preparation, the transethosome dispersion TE and the transethosomal chitosan gel TE-CHI were the formulations that provided the highest naftifine accumulation in the skin. Therefore, the transethosomal system formulated here could be a promising carrier for the topical delivery of naftifine. Additional studies assessing the safety and *in vitro* and *in vivo* antifungal activity are being studied.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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