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Research Article

Influence of a Combination of Chemical Enhancers and Iontophoresis on *In Vitro* Transungual Permeation of Nystatin

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Abstract. To promote transungual permeation of nystatin (NYST), molecule with high molecular weight, no water-soluble, amphoteric by iontophoresis. The synergic effect of the combination of cetylpyridinium chloride, CPC, or polyoxyethylene (20) sorbitan monooleate, TW80, and iontophoresis was investigated. *In vitro* permeation experiments were carried out through bovine hoof slices using vertical diffusion cells. A low current density (0.2 mA/cm^2) was applied by introducing Ag/AgCl electrodes in the donor (anode) and receptor (cathode) chambers. The donor phase consisted of a solution, a suspension, or gel-type vehicles containing NYST and surfactants in pH 5.6 HEPES buffer. The addition of CPC to NYST suspension (SOSP) produced a fivefold increase on the permeability of the bovine hoof membrane to the drug. The application of anodal iontophoresis further improved NYST flux. Conversely, NYST transungual permeation was not influenced by TW80 either in the passive diffusion or iontophoretic flux. Furthermore, the iontophoretic treatment does not appear to induce irreversible alterations to the hoof bovine membranes. The present work demonstrated the efficacy of iontophoresis as a treatment for different nail pathologies with large molecules very slightly soluble in water without irreversibly affecting the nail structure. A synergistic effect between CPC and iontophoresis was observed.

KEY WORDS: transungual permeation; bovine hoof membrane; iontophoresis; nystatin.

INTRODUCTION

Delivery of drug through the nail continues to receive significant attention due to the need for efficacious topical therapies for onychomycosis, common fungal infection of the nail. To date, many formulations are commercially available for the onychomycosis topical therapy as lacquers or solutions to apply on the nail but nonetheless, the problem of the low permeability of the substrate following ungual administration is not completely solved. A good nail penetration can be achieved by various methods: mechanical, chemical, and physical (1). Abrasion and avulsion (mechanical method) have been investigated by several researchers and can solve the problems although these were invasive techniques. These techniques allow exposing the whole nail matrix to the subsequent chemical or surgical matrixectomy, healing relapsing onychocryptosis, or supporting the treatment of long-term fungal infections of the nail. Chemical methods include the use of chemicals that can temporarily alter the barrier properties of the nail such as mercaptans/thiols (thioglycolic acid and 2-mercaptanethanol, N-acetyl-l-cysteine), keratolytic agents (urea and salicylic acid), and organic solvent (ethanol, isopropanol, propylene glycol, *etc.*). (2–4)

Photodynamic therapy and lasers may represent alternatives in the future (5). A more recent approach takes into account the hydration supported by occlusion to make nail matrix more permeable and elastic by increasing pores size (1). Among the physical methods used in topical field both for skin and nail treatments, the most common is the iontophoresis that consists in the application of electric field to drive compounds through the skin or other body structures such as the eye or nail. Iontophoresis acts on ionizable water-soluble products having positive or negative charge on the basis of electrorepulsion theory. The mechanisms, linked to the transport by iontophoresis, include electrophoresis (direct electrical field or Nernst-Planck effects) (6), electroosmosis (convective solvent flux) (7), and electropermeabilization (alteration of the membrane induced by the electrical field by improving its permeability) (8,9). Iontophoresis has proved effective both for ionic and electroneutral molecules (10,11).

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In the last years, different research papers demonstrated that iontophoresis significantly facilitated drug penetration/permeation through the nail with the help of an electric field. In particular, drug diffusion through the hydrated keratin of a nail may be improved by iontophoresis. This technique was successfully applied to increase transungual permeation of antifungal agents such as ciclopirox (3) and terbinafine, considered the most potent agents against dermatophytes, for treating topically onychomycosis and other nail diseases (12–16). Application of iontophoresis in *in vitro* and *ex vivo* experiments, after topical application, led to a significantly higher drug diffusion into the nail bed with respect to passive diffusion. A clinical study demonstrated that 40% of patients treated for 4 weeks with terbinafine and iontophoresis (current density, 0.1 mA/cm^2) showed an improvement in their health conditions *vs* 11% of those treated with only the drug by passive diffusion. It was demonstrated that the delivery of griseofulvin enhanced by eightfold with the help of iontophoresis, a success, since it has a low solubility in water and lipophilic characteristics (log P 2.0) with a limited ability to permeate into hydrophilic keratinous nail plate (17). A key role on increasing drug transungual permeation by iontophoresis is played by different parameters such as type of electrode, current density, co-ions, vehicle pH, charge, and drug concentration (3,11,18,19).

The aim of the present work was to find the optimal experimental conditions to promote transungual permeation of nystatin (NYST), a tetraene diene polyene macrolide (Fig. 1), applying iontophoresis. NYST was chosen as model of molecule with high molecular weight (926.09 Da), very slightly soluble in water (≈ 0.03%) with a minimum at pH 7, corresponding to the pI (isoelectric point), amphoteric with two ionizing groups, a carboxyl and an amino function with ionization constants (pK_a) estimated at 5.12 (pK_1) and 8.89 (pK_2) (20). Nystatin possesses different hydrophilic and lipophilic moieties: the hydrophilic area is composed of alcohols, carboxylic acid, and sugar whereas the lipophilic one consists of a chromophore of six conjugated double bonds.

The effect of the combination of cationic (cetylpyridinium chloride) or non-ionic surfactants (polyoxyethylene (20) sorbitan monooleate) and iontophoresis on NYST transport through fully hydrated bovine hoof membranes was investigated. Finally, the influence of the type of vehicle (solution, suspension, or gel-type) on *in vitro* transungual permeation of nystatin, by applying iontophoresis at low density current (0.2 mA/cm^2), was determined.

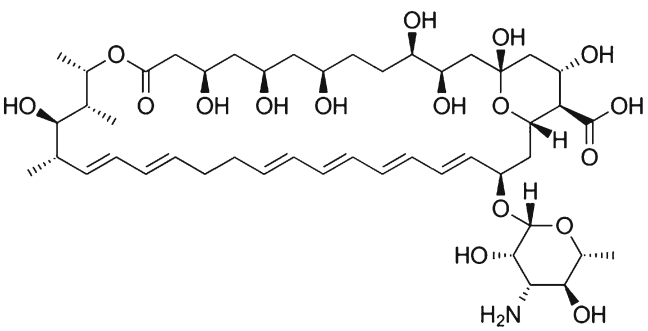

Fig. 1. Chemical structure of nystatin

MATERIALS AND METHODS

Materials

The following materials were used: nystatin (NYST, Sigma, Germany), cetylpyridinium chloride (CPC, Sigma, Italy), polyoxyethylene (20) sorbitan monooleate (Tween 80, TW80, Riedel-de Haen), hydroxypropylcellulose (HPC, Klucel JF, Hercules), and HEPES buffer (AppliChem GmbH, Germany). Methanol (MeOH, Carlo Erba, Italy), ethanol (Et, Carlo Erba, Italy), and dimethylformamide (DMF, Fluka, Germany) were all HPLC-grade. All other chemical and reagents used (sodium chloride, sodium hydroxide, sodium phosphate mono base monohydrate, disodium hydrogen phosphate dried) were analytical grade. All the solutions were prepared using high-purity deionized water (18 MΩ).

Formulations

The formulations under study (Table I) were prepared in pH 5.6, 25 mM HEPES buffer, and adjusted to a desired total ionic strength with sodium chloride providing a primary cationic (Na^+) and anionic (Cl^-) charge carrier. The suspension of NYST (SOSP) was prepared by introducing directly the drug in buffer solution; V1 and V2 formulations were prepared by dissolving surfactants in buffer solution and then adding NYST. Cetylpyridinium chloride, cationic surfactant, caused, in any case, the complete solubilization of the drug (micellar solution), whereas TW80, non-ionic surfactant, produced a partial solubilization of NYST.

G1–G4 gels were prepared by dissolving, at the first, CPC in HEPES buffer; then, appropriate amounts of HPC were added at room temperature under continuous stirring for 12 h; and finally, NYST was introduced in the dispersion. To prepare hydroalcoholic gels (G1/Et), HPC was dispersed in HEPES buffer containing 10% *w*/*w* of ethanol following the same procedure described above.

Measurement of viscosity was carried out on the semisolid formulations using HAAKE RheoStress viscometer RS1 and sensor system C60/4 (Thermo Fisher Scientific SpA, Italy). Tests were performed in triplicate at 32°C by applying increasing and decreasing values of the shear rate to detect possible thixotropy of the systems. The rheological profiles were determined with RheoWin Pro software. In all cases, pH of the formulations was 5.6.

NYST Delivery/Permeation Experiments

NYST In Vitro Permeation Through Bovine Hoof Membranes

In vitro permeation experiments were carried out through bovine hoof slices (thickness 90–120 μm) obtained following the procedure previously described (21). Modified Gummer vertical diffusion cells, consisted of donor and receiving chambers (respective volumes 2.0 and 5.8 ml) fastened together by a clamp, were used as apparatus. The

Table I. Composition of the Formulations Under Study

Formulations*	NYST (mg/ml)	CPC (% *w*/*w*)	TW80 (% *w*/*w*)	HPC (% *w*/*w*)	EtOH (% *w*/*w*)	NaCl (mM)
SOSP	0.5	–	–	–	–	50
V1	0.5	0.25	–	–	–	50
V2	0.5	–	0.125	–	–	50
G1	0.5	0.25	–	4.0	–	50
G2	1.0	0.25	–	4.0	–	50
G3	0.5	0.25	–	4.0	–	10
G4	0.5	0.25	–	4.0	–	100
G1/Et	0.5	–	–	4.0	10	50

*All formulations were prepared in pH 5.6, 25 mM HEPES buffer solution

hoof membranes, previously hydrated by immersions in water for 15 h to reach equilibrium in terms of hydration before the permeation tests, were placed between donor and receiving chambers, and the surface area available for permeation was 1.23 cm^2. The effective thickness of the hydrated membranes ranged between 90 and 120 μm.

To evaluate the influence of iontophoresis on transungual permeation of NYST, Ag/AgCl electrodes, most commonly used because they are reversible and resistant to changes in pH, were fixed in the donor and receptor chambers at a distance of 3 mm from the hoof membranes. IOMED Phoresor II Dose Controller System was used to apply a constant direct current (DC). The positively charged anode was introduced into donor chamber and the cathode into receptor chamber (anodal iontophoresis). Current density of 0.2 mA/cm^2 was applied to stimulate NYST penetration in the *in vitro* experiments. Two milliliters (for liquid vehicles) or 2 g (for semisolid vehicles) of formulations (SOSP, V1, V2, G1–G4, and G1/Et) were placed in the donor compartment. The receptor solution (catodal chamber) consisted of 25 mM HEPES buffer pH 7.4 plus 154 mM NaCl, stirred at 600 rpm, and maintained at 32°C. The solubility of NYST in the receiving phase at this temperature was 0.086 mg/ml. At predetermined intervals of time, receiving phase was completely withdrawn and replaced with the same volume of fresh buffer to ensure sink conditions. The amount of NYST permeated was determined by HPLC analysis. Since NYST in pH 7.4 aqueous solutions rapidly underwent a phenomenon of degradation, the samples of the receiving phase were immediately analyzed or maintained at −20°C before the analysis.

Two experimental protocols were used: protocol A consisted of passive permeation experiments for 6 h (control) and protocol B consisted of iontophoretic treatment for 4 h applying constant direct current of 0.2 mA/cm^2 (B1-1°period), followed by 2 h of passive permeation (B2-2°period). In any case, the tests were replicated at least five times and the experiments were performed completely in the dark.

Linear regression analysis of pseudo steady-state diffusion data allowed calculation of J, the steady-state flux (given by $Q/A \cdot t$, where Q is the amount of permeant diffusing across the area A in time t) normalized to the thickness of the biological substrate and amount of drug permeated at the end of the permeation experiment.

NYST In Vitro Release Through Cellulose Acetate Membranes

In vitro release of NYST from the V1-G1-G1/Et formulations through cellulose acetate membranes (SpectraPore3, MWCO3500, Spectrum®, NL) was investigated using vertical Gummer cells (22) with an effective diffusion area of 1.23 cm^2. Five milliliters of 25 mM HEPES buffer (pH 7.4) plus 154 mM NaCl, thermostated at 32°C and stirred at 600 rpm, were used as the receptor medium. Two milliliters or 2 g of the formulations under study (donor phase) were put in contact with the membrane. At predetermined time intervals, samples of the receiving phase were withdrawn for analysis and replaced with an equal volume of fresh buffer to maintain sink conditions. The amount of NYST released was determined by HPLC. Each release test was replicated at least three times.

Analytical Method

Quantitative determination of NYST in the samples was carried out by HPLC. The apparatus consisted of a Shimadzu LC-10AD system with an UV SPD-10AV detection and a software Cromatoplus analyzer. The injection valve was a Rheodyne with a capacity of 20 μl. A Phenomenex C18 analytical column (4.60 × 100 mm, Luna, 3 μ) was employed. The mobile phase consisted of MeOH:water:DMF (60:30:10) mixture (flow rate 1.0 ml/min); the retention time and the detection wavelength were 7.5 min and 305 nm, respectively. The limit of determination and quantification were 6.9 and 2.0 ng/ml, respectively. The amount of drug in each sample was determined from standard curves, obtained by plotting the concentration of known solutions *vs* the corresponding peak areas of HPLC chromatograms.

Statistical Analysis

All data are the average of five determinations ± standard error (SE). Significant differences between permeation parameters were assessed by GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The evaluation included the calculation of the means and standard errors and group comparisons using Student's two-tailed unpaired t test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Table II summarizes steady-state flux (μg cm^{-2} h^{-1}) of NYST for the different treatments and the different formulations. The flux was calculated during passive permeation (protocol A), iontophoretic treatment (protocol B1), and post-iontophoretic treatment (protocol B2).

At first, this work was directed to understand the transungual kinetic of NYST from a suspension: SOSP produced a flux of 0.14 ± 0.011 μg cm^{-2} h^{-1}, during the passive permeation; applying iontophoresis raised NYST transungual permeation up to ~4.5-fold ($J = 0.63 \pm 0.024$ μg cm^{-2} h^{-1}).

The addition of CPC to SOSP (V1) produced an increase (approximately fivefold) on the NYST permeability of the bovine hoof membrane compared to that of the suspension. CPC promoted the passive diffusion of NYST probably because of the solubilizing ability of the surfactant; CPC was used at a concentration above CMC (0.04% *w/w*) and allowed a complete solubilization of drug in pH 5.6 HEPES buffer solution; the application of anodal iontophoresis (protocol B1) improved further NYST flux passing from 0.76 ± 0.027 to 2.12 ± 0.079 μg cm^{-2} h^{-1} during 4 h of treatment (Table II), indicating that the drug and the cationic surfactant did not have a competitive mechanism.

On the contrary, NYST transungual permeation was not influenced by the presence of TW80: there are no significant statistically differences either in the passive diffusion or iontophoretic flux with respect to SOSP ($J = 0.25$ *vs* 0.14 μg cm^{-2} h^{-1} and $J = 0.63$ *vs* 0.45 μg cm^{-2} h^{-1}, respectively). Furthermore, when TW80 was added to the formulation, the iontophoretic flux increased from 0.25 to 0.45 μg cm^{-2} h^{-1}).

The differences among the vehicles under study are also well highlighted by the amount of drug permeated at the end of the permeation studies using the different experimental protocols. When the iontophoresis conditions were adopted, the amount of NYST permeated in presence of cationic surfactant (V1) was remarkably higher than that when non-ionic surfactant was added in the donor phase (V2) (Table III).

At this point, NYST was introduced in a hydrogel that can provide an electroconductive base and make application easier as a hydrogel pad can adapt to the application site. The passive diffusion flux of NYST through the bovine hoof membrane from the HPC hydrogel containing CPC as a chemical enhancer (G1) was 0.34 ± 0.020 μg cm^{-2} h^{-1} about twofold lower than that obtained by V1 (0.76 ± 0.027 μg cm^{-2} h^{-1}). All gels exhibited a Newtonian behavior, with linear relationship between shear rate (D) and shear stress (tau). No thixotropy phenomenon was observed and the viscosity of all gel-type formulations under study was in the range from 550 to 780 mPa·s. The increase in the vehicle viscosity reduced the diffusion rate of drug through the gel matrix and consequently the transungual permeation. In fact, the release rate of NYST through the cellulose acetate membrane was 2.57 ± 0.24 μg cm^{-2} h^{-1} for G1 against 25.31 ± 1.73 μg cm^{-2} h^{-1} for V1; besides, after 6 h, NYST amount released from G1 was about nine times less than that of V1 (18.68 ± 1.14 *vs* 165.58 ± 15.35 μg cm^{-2}).

The application of current (protocol B1) improved the flux of NYST from G1 (2.08 ± 0.107 μg cm^{-2} h^{-1}) to reach that of V1 (2.12 ± 0.079 μg cm^{-2} h^{-1}, protocol B1) probably due to a best electroconductivity that partially counteracts the effect of the high viscosity on the diffusion of the drug in the vehicle.

The effect of ionic strength on penetration was investigated by modifying NaCl concentration in the donor solution (10, 50, or 100 mM) since the concentration of electrolytes may compete with the drug ion. Increasing NaCl concentration from 10 to 100 mM in the donor phase improved the drug permeation under effect of the application of the current from 1.63 ± 0.12 to 3.29 ± 0.18 μg cm^{-2} h; otherwise, no change of the passive diffusion was observed (Fig. 2). It is evident that there was a direct relationship between ionic strength and permeation enhancement with significant statistical differences, while a change in NYST concentration from 0.5 to 1.0 mg/ml (Table II) did not affect the iontophoretic delivery of drug: the amount of NYST permeated was 8.81 ± 0.44 and 8.79 ± 1.25 μg/cm^2 for 0.5 and 1.0 mg/ml formulations, respectively. At higher drug concentration, the current is mainly carried by electrolytes that are small, fast, and mobile molecules at the expense of NYST.

Table II. Steady-State Flux of NYST Through Hoof Bovine Membranes Obtained from the Formulations Under Study (mean ± S.E., $n = 5$)

Formulations	Flux (μg cm^{-2} h^{-1})		
	Protocol A Passive transport	*Protocol B*	
		B1—iontophoretic transport	*B2*—post-iontophoretic transport
SOSP	0.14 ± 0.011	0.63 ± 0.024*	0.40 ± 0.056
V1	0.76 ± 0.027	2.12 ± 0.079*#	1.30 ± 0.057
V2	0.25 ± 0.024	0.45 ± 0.010	0.41 ± 0.003
G1	0.34 ± 0.020	2.08 ± 0.107*#	1.26 ± 0.137
G2	0.45 ± 0.021	1.97 ± 0.491*#	1.31 ± 0.379
G3	0.33 ± 0.022	1.63 ± 0.120*#	1.06 ± 0.097
G4	0.36 ± 0.035	3.29 ± 0.180*#	2.06 ± 0.135
G1/Et	0.31 ± 0.024	1.63 ± 0.116*#	1.08 ± 0.089

*Significantly different from the passive transport (protocol A), $p < 0.05$
Significantly different from SOSP applying iontophoresis, $p < 0.05$

Table III. Amount of NYST Permeated Through Hoof Bovine Membranes After 4 h (mean ± S.E., $n = 5$)

Formulations	NYST permeated (μg cm^{-2})	
	Protocol A Passive permeation	*Protocol B1* Iontophoretic transport
SOSP	0.51 ± 0.038	2.50 ± 0.094*
V1	2.90 ± 0.128	7.94 ± 0.312*
V2	0.99 ± 0.069	1.68 ± 0.048
G1	1.61 ± 0.129	8.81 ± 0.441*
G2	2.11 ± 0.080	8.79 ± 1.150*
G3	1.55 ± 0.104	6.34 ± 0.499*
G4	1.67 ± 0.147	12.29 ± 0.698*
G1/Et	1.44 ± 0.042	6.74 ± 0.315*

*Significantly different from the passive transport (protocol A), $p < 0.05$

The substitution of the surfactant with ethanol (10%, G1/Et), as co-solvent/promoter to solubilize drug in the formulation, appeared not to have a significant effect both on passive permeation of NYST and under iontophoretic conditions. The 10% ethanol was not sufficient to solubilize the drug and, in any case, played a negative role on dehydration of the bovine hoof membrane that contributes to reduce the permeation. Although ethanol is an effective skin permeation enhancer, it does not have a similar effect on the nail. Ethanol acts on the stratum corneum by altering intercellular lipids; however, the lipid content of the nail comprises just 0.15–0.76% of its total weight (23). Any increase in drug solubility engendered by adding an organic solvent to a formulation to improve transungual drug delivery may be counteracted by the reduction in nail hydration and permeability caused by the solvent itself. Smith et al. (24) studied the effect of organic solvents EtOH, PPG, and PEG on the barrier properties of nail by demonstrating that permeant partitioning into and transport across the nail were shown to decrease as the concentration of the organic solvent in the binary solvent system increased.

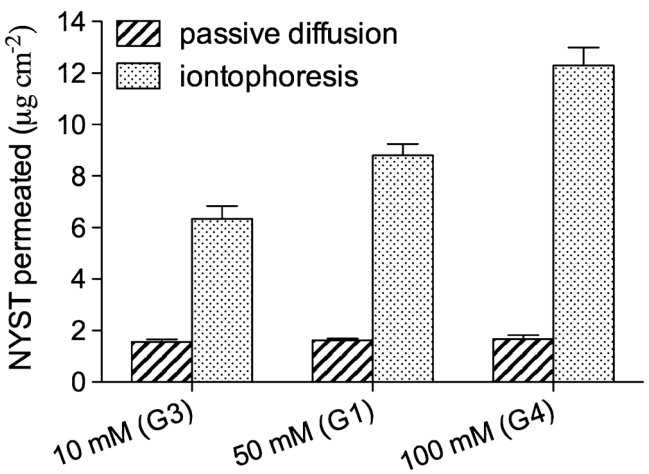

Fig. 2. Effect of ionic strength on nystatin permeated through bovine hoof membrane for passive diffusion and after iontophoretic treatment

The last part of the work was dedicated to understand whether the iontophoresis process caused permanent damages to the tissue once the current supply was stopped.

Data, summarized in Table II, highlighted that, when the current was turned off (protocol B2), NYST molecules persisted to permeate the membrane although the flux was decreasing after stopping of iontophoresis. This could depend on either an alteration of the biological matrix or the existence of a drug reservoir in the membrane. As example, Fig. 3 illustrates the transungual permeation profiles of NYST from SOSP and V1 (CPC) formulations before and after stopping of current.

DISCUSSION

This work was aimed at promoting transungual permeation of nystatin, an amphoteric macromolecule, using the most suitable combination between chemical and physical methods. Nystatin was chosen as model drug with high molecular weight (926.09 Da), which makes it very difficult to pass through a barrier by passive diffusion, very slightly soluble in water, but amphoteric with two ionizing groups. The search of a technique that increases transungual permeation of this type of molecules would give a great scientific contribution to this field, despite NYST was not easy to handle since it quickly undergoes a degradation phenomenon in aqueous solution. The degradation depends on the pH of the aqueous solution: it is known (20) that nystatin is optimally stable in aqueous solution at pH from 5 to 7 (phosphate and citrate buffer), when held at 37°C. In this work, the stability of drug in the formulations at pH 5.6 and in the receiving phase at pH 7.4 for the time of the permeation experiments has been verified. NYST in the formulations under study (pH = 5.6) remained completely stable during the tests while NYST at pH 7.4 was stable in the first hour at all tested temperatures (4, 20, and 32°C). On the contrary, after

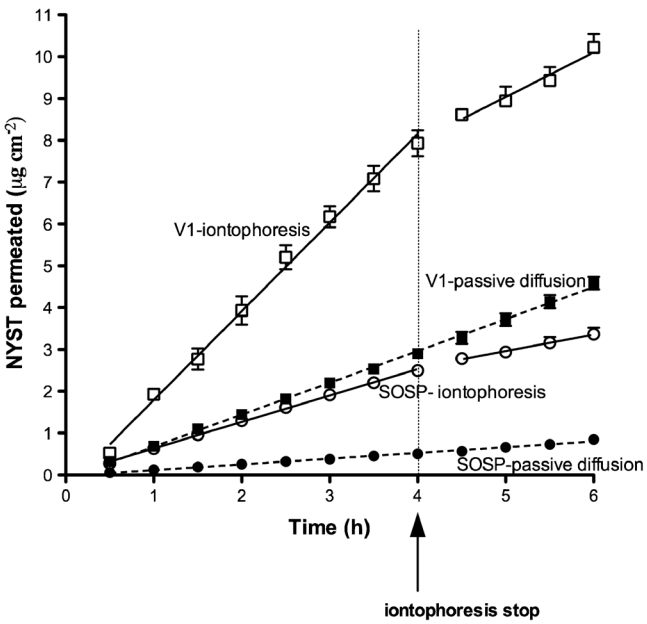

Fig. 3. Permeation profiles of nystatin from SOSP and V1 formulations through bovine hoof membrane stopping iontophoretic treatment after 4 h

6 h, drug recovery was 87, 82, and 76% at 4, 20, and 32°C, respectively. To overcome this problem, the samples of the receiving phase just after withdrawal were immediately analyzed or frozen at −20°C.

As an enhancer, two surfactants, one cationic (CPC), and one non-ionic (TW80) were chosen, first of all, to increase the drug solubility in the aqueous vehicle and, then, to study the role of the charge on the iontophoretic process. Both CPC and TW80 were used above CMC but only CPC (V1), that produced complete dissolution of the drug (micellar solution), demonstrated its enhancement effect by increasing NYST passive diffusion through bovine hoof membranes compared to that obtained by SOSP. On the other hand, TW80 appeared not to modify the NYST transungual permeation and, moreover, not to help the solubilization of the drug in the formulation; in fact, the addition of 0.125% TW80, to obtain vehicle V2, produced a small increase in pH 5.6 HEPES buffer NYST solubility (from 0.05 to 0.07 mg/ml at room temperature, for SOSP and V2 respectively), which was not further increased by doubling the surfactant concentration; consequently, it did not positively affect either the solubilization process or the interaction with the barrier. These data confirm the role of solubility in increasing thermodynamic activity and the permeation of drug across biological membranes (13,25).

In addition, it could be assumed that the surfactant, in particular CPC, due to its amphoteric nature and its cationic form, binds to the keratin, thus enabling NYST partially positively charged at the pH 5.6 of the formulation to diffuse through the nail matrix and to reach the receiving phase; the surfactant could improve transungual permeation entering in competition with the antifungal agent in the form of an acid salt for binding to keratin of the various layers of the nail that are negatively charged (26). Moreover, it has been reported that CPC included in a topical nanoemulsion at 0.25% *w/v* for the treatment of onhycomycosis was able to diffuse laterally from the nail edge across the nail bed and matrix and did not negatively influenced the formulation tolerability in terms of skin irritation and any serious adverse events was noticed (27,28).

These formulations (SOSP, V1, and V2) were used, as donor phase, in the iontophoretic study. HEPES buffer is chosen for its common use in iontophoresis research as it is zwitterionic at pH 7.4 with a reduced ability of charge carrying. This aspect is crucial when you want to discriminate the role of osmotic effect in relation to the ion concentrations because the iontophoretic delivery of the drug will be reduced by these buffer ions as they will compete with the drug for carrying the current noticed (29).

Moreover, HEPES buffer has been used to prevent the formation of a phosphate deposit on the electrode and to improve the sensitivity of the analytical method for quantitative analysis of NYST. The conditions of iontophoresis in term of current density (0.2 mA/cm^2) and ionic strength (donor phase, 50 mM; receiving phase, 154 mM) were chosen on the basis of literature data (19,30). Nair et al. (14) demonstrated that transungual permeation of terbinafine (TBF) was proportionally enhanced with an increase in applied current density from 0.1 to 1.0 mA/cm^2 as well as current duration (15, 30, 45, 60 min) but the amount of drug permeated, besides at low current density (0.1 mA/cm^2), was significantly higher than that in the MIC (14,31) and in any case at 0.25-mA/cm^2 current density, TBF flux was increased of about 12 times.

During the present study, the ionic strength of receiver was maintained at 154 mM close the physiological value as well as pH (7.4) (32) while for the donor phase, 50 mM was selected, considering that an ionic strength of at least 50–100 was needed to have an optimal electric current conduction across nails as found by Murthy et al. (10). To choose these conditions, it has been taken into consideration that there is a critical concentration range over which the ionic competition between drug and electrolytes negatively influences the current transport, mainly carried by electrolyte co-ion (10). On the contrary, the electrode chambers (in particular, at the anode) must contain a definite amount of electrolyte to maintain the electrochemistry of the Ag/AgCl couple.

About the pH, we chose pH 7.4 (physiological pH) for receiving phase and pH 5.6 for donor phase. The pH of the donor phase was chosen taking into account that donor phase influences the charge density on the biological barrier (11) and, since the keratin in the nail plate has an isoelectric point (pI) of ~5, at pH 5.6 hoof bovine keratin membrane was negatively charged, while NYST was partially positively charged. These pH conditions were chosen to promote NYST anodal iontophoretic transport at pH above 5 compatible with the nail permselectivity depending on pH. Nail contains a much lower lipid amount than the skin and it does not contain follicles, important access for cutaneous iontophoresis. The hydrated nail is similar to a porous hydrophilic hydrogel and its resistance to diffusion is provided by the keratin fibers suspended in a continuous aqueous medium (14,33). NYST, with its positive charge, will be rejected by positive electrode (anode) and anodal iontophoresis will be effective to promote NYST transungual permeation.

In our study, the use of anodal iontophoresis increased the drug transungual transport already when the suspension is used as a vehicle (SOSP), facilitating the permeation of the solubilized and ionized drug fraction. The greatest contribution of iontophoretic technique to NYST transungual permeation was obtained using the V1 formulation with CPC, suggesting that the formation of cationic micelles with CPC could influence iontophoretic transport promoting the interaction with negatively charged biological membranes.

When the current was applied in presence of V2 formulation containing TW80, there was an increase of 1.8-fold in NYST flux (0.45 *vs* 0.25 μg cm^{-2} h) and in the amount of drug permeated (0.99 *vs* 1.68). These values are lower than those obtained after iontophoretic treatment of SOSP formulation even though without statistically significant differences. The surfactant seems to have a shielding effect on the dissociated NYST in the experimental conditions chosen with a lesser influence on the electric field.

The combination CPC/iontophoresis appears suitable to promote the transport NYST through the ungual barrier. Increasing drug aqueous solubility seems critical in developing an efficient transungual iontophoretic delivery system of poorly water-soluble molecules.

It is known that the nail under normal circumstances is a negatively charged, cation-permselective membrane. Under the influence of an electric field, therefore, the transport of cationic substances across the hydrated nail should be enhanced by anodal iontophoresis. Moreover, a convective,

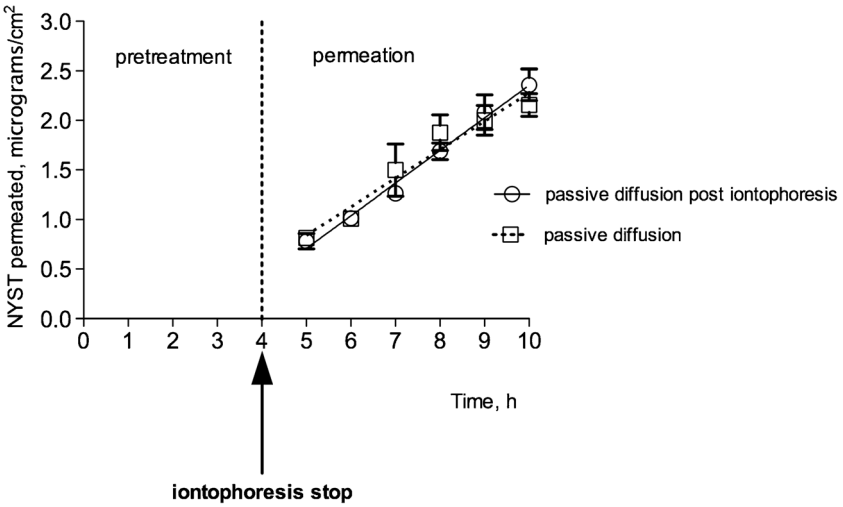

Fig. 4. Transungual permeation profile of nystatin by passive diffusion with (circle) and without (square) iontophoretic pre-treatment

electroosmotic flux proceeds in the anode-to-cathode direction, supplementing cationic transport during iontophoresis and allowing the enhanced transport of neutral polar substances above all in the case of anodal iontophoresis (34). It is known that changes in ionic strength can modify ion distribution in the pores of the nail, besides when the iontophoresis conditions were used.

In our paper, the effect of ionic strength was determined under "asymmetric conditions" as reported by Smith et al. (32): the donor ionic strength was changed while the one of the receiver was maintained at the physiological value in order to resemble *in vivo* conditions. A direct relationship between NYST transungual permeation and ionic strength was observed (Fig. 2), suggesting a considerable contribution of the electroosmotic phenomenon, demonstrated also by the irrelevance of an increase in drug concentration on producing any change in the flux values.

The last part of the work was dedicated to understand if the iontophoresis process caused permanent damages to the tissue once the current supply was stopped.

The effect of iontophoresis on the tissue structure was studied performing the permeation experiments from G4 formulation, where the effect of iontophoresis was more remarkable, according to the following experimental protocol: the fully hydrated ungual substrate was pre-treated with iontophoresis (anodal, current density: 0.2 mA/cm^2) for 4 h using pH 5.6 HEPES buffer solution (100 mM) without drug, as donor phase; after this period, buffer solution was replaced with G4 formulation, as donor phase, and the passive permeation was followed for 6 h on the same bovine hoof membrane. At the same time, an experiment under the same experimental conditions (pH 5.6 HEPES buffer solution, as donor phase, pre-treatment for 4 h, then passive diffusion from G4 formulation for 6 h) was performed without application of iontophoresis. No difference in the NYST permeation profile was observed comparing the passive diffusion with and without iontophoretic pre-treatment, as well highlighted in Fig. 4; no statistically significant difference between flux values ($J = 0.33 \pm 0.02$ *vs* 0.29 ± 0.04 μg/cm^2 h, respectively) and amount of NYST permeated (2.35 ± 0.15 *vs* 2.16 ± 0.11 μg/cm^2, respectively) was demonstrated. Anyway, there is a little difference in the amount of NYST permeated in these conditions with respect to the ordinary passive diffusion without 4-h pre-treatment period (1.67 ± 0.147 *vs* 2.16 ± 0.11 μg/cm^2), probably due to an increased membrane hydration for a prolonged contact with the buffer solution, independent of the application of the electric field. As reported by Hao et al. (3), it could be hypothesized a direct correlation between nail resistance and concentration of the solutions in contact with the membranes. In any case, the current application did not seem to modify the permeability of ungual membrane by suggesting that the iontophoretic treatment does not provoke irreversible alterations to the hoof bovine membranes.

The two experiments performed for this goal appeared to suggest a reversible interaction between the drug molecules and the keratin matrix of the bovine hoof that would then act as a reservoir to release the drug over the time considering that no damage to the tissue has been demonstrated by iontophoretic treatment. The role of iontophoresis on nail reservoir effect was proposed also by other authors, for example in the case of transungual permeation of terbinafine (14).

CONCLUSIONS

The present work demonstrated that the iontophoretic technique could represent an effective strategy for treatment of different pathologies of the nail promoting the transungual permeation of large molecules with high molecular weight and very slightly soluble in water without irreversibly affecting the nail structure. Indeed, the application of low current density (0.2 mA/cm^2) for a short period of time (4 h) could significantly enhance the drug transport across the membranes creating a depot effect, which will contribute to a further release of the drug when the electric field was switched off, reducing the need of frequent dosing. Moreover, the presence of a cationic surfactant such as CPC showed a synergistic effect with the iontophoretic procedure. The opportunity to exploit these conditions may contribute to improve the efficacy of topical nail therapy, which is impaired

by low permeation of drug molecules across the highly keratinized nail plates.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

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