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Penetration enhancer-containing vesicles (PEVs) as carriers for cutaneous delivery of minoxidil

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ABSTRACT

The aim of this work was to evaluate the ability of a few different penetration enhancers to produce elastic vesicles with soy lecithin and the influence of the obtained vesicles on *in vitro* (trans)dermal delivery of minoxidil. To this purpose, so-called Penetration Enhancer-containing Vesicles (PEVs) were prepared as dehydrated–rehydrated vesicles by using soy lecithin and different amounts of three penetration enhancers, 2-(2-ethoxyethoxy)ethanol (Transcutol®), capryl-caproyl macrogol 8-glyceride (Labrasol®), and cineole. Soy lecithin liposomes, without penetration enhancers, were used as control. Prepared formulations were characterized in terms of size distribution, morphology, zeta potential, and vesicle deformability.

The influence of PEVs on (trans)dermal delivery of minoxidil was studied by *in vitro* diffusion experiments through newborn pig skin in comparison with traditional liposomes and ethanolic solutions of the drug also containing each penetration enhancer. A skin pre-treatment study using empty PEVs and conventional liposomes was also carried out.

Results showed that all the used penetration enhancers were able to give more deformable vesicles than conventional liposomes with a good drug entrapment efficiency and stability. *In vitro* skin penetration data showed that PEVs were able to give a statistically significant improvement of minoxidil deposition in the skin in comparison with classic liposomes and penetration enhancer-containing drug ethanolic solutions without any transdermal delivery. Moreover, the most deformable PEVs, prepared with Labrasol® and cineole, were also able to deliver to the skin a higher total amount of minoxidil than the PE alcoholic solutions thus suggesting that minoxidil delivery to the skin was strictly correlated to vesicle deformability, and therefore to vesicle composition.

1. Introduction

Minoxidil is the most widely used drug for the treatment of male and female hair loss and topical minoxidil is effective in reversing the progressive miniaturization of hair follicles associated with androgenic alopecia, by direct effect on the proliferation and apoptosis of dermal papilla cells. However, chronic dosing studies have demonstrated that the effect of minoxidil is not permanent, and cessation of treatment will lead to hair loss in 4–6 months.

Conventional topical minoxidil formulations consist of propylene glycol–water–ethanol solutions (Tata et al., 1995; Messenger, 2004). However, twice-daily applications, recommended in proper use, might be accountable for severe adverse reactions (e.g. scalp dryness, irritation, burning, redness, allergic contact dermatitis) (Pavithran, 1993). To minimise side effects and improve therapeutic efficiency, new dermatological preparations for topical application are required.

Skin represents an ideal route of drug administration in terms of accessibility and ease of application but the main obstacle to (trans)dermal drug delivery is the low penetration of drugs through the cutaneous barrier provided by stratum corneum (SC). Several approaches have been used to enhance skin penetration of drugs. Among the most popular possible techniques, vesicular systems have been intensively studied as carriers for skin delivery of drugs. Traditional liposomal formulations, compared to conventional dosage forms, have shown *in vitro* an enhanced cutaneous drug accumulation allowing a reduction of the dose applied onto the skin (Mezei and Gulasekharam, 1980, 1982; Touitou et al., 1994; Fresta and Puglisi, 1996; Barry, 2001; Cevc and Blume, 2004; Sinico et al., 2005; Mura et al., 2007).

In the last two decades, new classes of lipid vesicles were introduced by different researchers. In 1992, Cevc et al. introduced the first generation of the highly deformable, elastic liposomes, referred as Transfersomes®. They consist of phospholipids and a

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surfactant molecule, the so-called "edge activator", which destabilizes lipid bilayer and increases its deformability. A second generation of elastic vesicles, mainly consisting of non-ionic surfactants, was introduced in 1999 by van den Bergh et al. (1999). Touitou et al. (2000) developed ethosomes, new soft vesicular carriers mainly consisting of phospholipids, ethanol and water. More recently, Verma and Fahr's group introduced invasomes, composed of phosphatidylcholine, ethanol and a mixture of terpenes as penetration enhancers (Verma, 2002; Dragicevic-Curic et al., 2008). Although most studies have shown that all these vesicles are able to enhance transdermal drug delivery, they have also been reported to improve drug skin deposition with a higher effectiveness than conventional vesicles (El Maghraby et al., 2001; Trotta et al., 2002, Elsayed et al., 2006).

Several molecules have been suggested as an edge activator for deformable vesicle formation. An edge activator is often a single chain surfactant, having a high radius of curvature, which is able to destabilize lipid bilayers increasing their deformability. Sodium cholate, different Spans and Tweens, and potassium glycyrrhinizate were used (Cevc et al., 1996; El Maghraby et al., 1999, 2000; Trotta et al., 2002). An alternative approach to fluidize the liposomal membrane consists in liposomal incorporation of high amount of ethanol giving rise to ethosomes, capable of highly improving transdermal drug delivery (Touitou et al., 2000; López-Pinto et al., 2005; Paolino et al., 2005). Propylene glycol has also been suggested in new vesicular formulations as an alternative to ethanol (Elsayed et al., 2007). New elastic liposomes were also prepared by incorporating in the lipid bilayer a few permeation enhancers, e.g. oleic acid and limonene (El Maghraby et al., 2004), and a mixture of terpenes and ethanol (Verma, 2002; Dragicevic-Curic et al., 2008).

Results obtained in the literature by using deformable liposomes and niosomes are conflicting as already shown with classic vesicular formulations. These results depend on several factors among which composition and therefore the edge activator choice is of particular importance.

Therefore, in the attempt to find vesicular formulations able to optimize cutaneous delivery of minoxidil, during this work we started a research on new liposomal systems prepared by using in their composition a few penetration enhancers (PEs) which differ for chemical structure and physicochemical properties. Therefore, so-called Penetration Enhancer-containing Vesicles (PEVs) were prepared by using soy lecithin and different amounts of three penetration enhancers, 2-(2-ethoxyethoxy)ethanol (Transcutol®), capryl-caproyl macrogol 8-glyceride (Labrasol®), and cineole.

The aim of this work was to evaluate the ability of the different penetration enhancers to produce elastic vesicles with soy lecithin and to study the influence of the PEVs on (trans)dermal delivery of minoxidil. Liposomal formulations were prepared as dehydrated–rehydrated vesicles (DRVs) using soy lecithin and dicetylphosphate as main components. *In vitro* diffusion experiments were performed through newborn pig skin in comparison with traditional liposomes and ethanolic solutions of the drug also containing each PE. A skin pre-treatment study using empty PEVs and conventional liposomes was also carried out.

2. Materials and methods

2.1. Materials

Soy lecithin (SL) was obtained from Galeno (Potenza, Italy). Minoxidil (MW 209.25, 99% purity) was purchased from Farma Labor (Bari, Italy). Dicetylphosphate (DCP) and cineole were purchased from Aldrich (Milan, Italy). 2-(2-Ethoxyethoxy)ethanol (Transcutol®) and capryl-caproyl macrogol 8-glyceride (Labrasol®) were a gift from Gattefossè (Saint-Priest, France). All the products and solvents were of analytical grade.

Table 1
Composition of minoxidil vesicular formulations (mg/ml).

Components	Control	PEV-1	PEV-2	PEV-3
Soy lecithin	60	60	60	60
Dicetylphosphate	5.4	5.4	5.4	5.4
Minoxidil	50	50	50	50
Labrasol		20		
Transcutol			20	
Cineole				10

2.2. Solubility studies

The solubility of minoxidil was determined in each penetration enhancer. An excess of drug was added to the medium in screw capped tubes (10 ml) and stirred at 25 °C for 48 h. Each sample was centrifuged and 0.2 ml of the clear supernatant were diluted with methanol and analysed by high performance liquid chromatography as described below.

2.3. Vesicle preparation

Composition of minoxidil carriers is reported in Table 1. Dehydrated–rehydrated vesicles (DRV) were prepared according to previously described methods (Mura et al., 2007). Soy lecithin, DCP, minoxidil and the penetration enhancer (for PEV formulations) were dissolved in chloroform. Then, lipid–drug mixture was deposited as a thin film in a round-bottom flask by roto-evaporating (Rotavapor Büchi R110, Switzerland) the chloroform under vacuum, which was applied for 1 h to ensure total removal of solvent traces. The film was hydrated for 1 h at room temperature by distilled water under mechanical stirring. Vesicle suspension was sonicated in a Soniprep 150 apparatus (MSE Crowley, Beckenham, UK) in 25 cycles of 10 s "on" and 10 s "off". Liposomal dispersions, were freezed at −20 °C for 12 h and then freeze-dried overnight using a Criotecnica freeze-drier apparatus (MM Cota Company, Roma, Italy) with 60 mmHg operative pressure at −80 °C. Freeze-dried vesicles were stored at 25 °C for 30 days. Then, they were reconstituted by controlled rehydration in distilled water and mechanically shaken by a vortex (Velp scientifica, Italy) for 10 min.

2.4. Incorporation efficiency (E%)

Incorporation efficiency (*E%*), expressed as a percentage of the total amount of minoxidil found in the studied formulations at the end of the preparation procedure, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with Triton X-100. The analyses were carried out with a liquid chromatograph Alliance 2690 (Waters Corp, Milford, MA), equipped with a photodiode array detector and a computer integrating apparatus (Millennium 32), by reverse phase absorption chromatography using a Nova-Pack C18 (60 Å, 4 μm) HPLC cartridge (Waters, Vimodrone Milano, Italy). The mobile phase was a mixture of methanol and water (80:20, v/v). The sample volume injected was 50 μl. Detection was performed at 231 nm. The flow rate was 2.2 ml/min. Retention time of minoxidil was 1.4 min. The detection limit of minoxidil was 10 ng/ml. For concentrations in the range 0–50 μg/ml a good linearity was obtained ($R^2 = 0.998$; $p < 0.001$).

2.5. Vesicle characterization

Vesicles were characterized by transmission electron microscopy (TEM) and optical or light polarised microscopy

for vesicular formation and morphology. A drop of vesicle dispersion was applied to a carbon film-covered copper grid and was stained with a 1% phosphotungstic acid. Then, samples were examined with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV. Optical and light polarised micrographs were obtained using an optical microscope Zeiss Axioplan 2 at 25 °C. The temperature was kept constant ±1 °C by a hot stage device connected to a thermostatic bath.

Average diameter and polydispersity index (PI) of all the samples were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3 apparatus (Malvern, UK). Instrument detects the scattering information at 173 °C and 25 °C. This is known as backscatter detection. It is important to note that this mean size is an intensity mean. Sample mean size was detected as bimodal distribution of sizes. The polydispersity index (PI) was used as a measure of the width of the size distribution. PI less than 0.4 indicates a homogenous and monodisperse population.

Zeta potential was measured as the particle electrophoretic mobility means of laser microelectrophoresis in a thermostated cell.

All the samples were analysed 24 h after their preparation. They were diluted with distilled water before the measurements. In particular, for both particle size and zeta potential determinations, 50 μl of each vesicle dispersion was diluted to 10 ml with distilled water. Each sample was measured three times and results are expressed as mean ± standard deviation.

2.6. Deformation index determination

Comparative measurement of deformability of liposome bilayer with different penetration enhancers was carried out by extrusion measurement. The vesicle dispersion was extruded at constant pressure through polycarbonate filters of definite pore size (Nucleopore®, 50 nm), using an extrusion device Liposofast® (Avestin). The deformability of vesicle was expressed in terms of deformation index (DI) according to Eq. (1):

$$\mathrm{DI} = J\left(\frac{d_0}{p}\right)^k \left(\frac{1}{|d_1 - d_0|}\right) \quad (1)$$

where J is the amount of suspension recovered after extrusion, d_0 and d_1 are the mean diameter of vesicles before and after extrusion, p is the pore size of the membrane, and k is an amplification factor.

2.7. In vitro skin penetration and permeation studies

Experiments were performed non-occlusively using vertical diffusion Franz cells with an effective diffusion area of 0.785 cm^2, using newborn pig skin. The experiments were performed by using the skin of newborn Goland-Pietrain hybrid pigs. The pigs (~1.2 kg) died of natural causes a few h after birth, and they were provided to us by a local slaughterhouse.

The skin, previously frozen at −18 °C, was pre-equilibrated in PBS solution at +25 °C for 2 h before the experiments. Before use, the lyophilized minoxidil loading vesicles with and without enhancer (control) were rehydrated with distilled water and mechanically shaken until a milky suspension was obtained. Newborn pig skin specimens were sandwiched securely between donor and receptor compartments of the Franz cells, with the epidermis side facing the donor compartment. The receptor compartment was filled with 5.5 ml of hydroalcoholic solution (water/ethanol 1/1, w/w) which was continuously stirred with a small magnetic bar and thermostated at 37 ± 1 °C throughout the experiments to reach the physiological skin temperature (i.e. 32 ± 1 °C). 100 μl of either minoxidil entrapped vesicle with or without (control) PE were placed onto the skin surface (n = 6 kin specimens per tested formulation). At regular intervals of 2 h up to 8 h, the receiving solution was withdrawn and analysed by HPLC for its minoxidil content. Samples were replaced with an equivalent volume of hydroalcoholic solution to ensure sink conditions.

After 8 h, the skin surface of specimens was washed 3 times with 1 ml of distilled water then dried with filter paper. The stratum corneum (SC) was removed by striping with an adhesive tape Tesa® AG (Hamburg, Germany). A piece of the adhesive tape was firmly pressed on the skin surface and rapidly pulled off with one fluent stroke. Ten stripping procedures were performed consecutively. The epidermis was separated from the dermis with a surgical sterile scalpel. The 10 tape strips, epidermis, and dermis were cut and placed each in a vial with 3 ml of methanol and then sonicated for 4 min in an ice bath to extract the drug. The tape and tissue suspensions were centrifuged for 10 min at 10,000 rpm, then the supernatants were filtered and assayed for their minoxidil content by HPLC as described above.

Hydroalcoholic solutions of minoxidil modified by addiction of each free PE at the same amount used in the PEVs formulations were prepared. 100 μl of these solutions was applied onto the skin surface and the *in vitro* experiments were carried out as described above.

Pre-treatment studies were performed applying overnight 100 μl of empty PEVs and soy lecithin liposomes (control) onto the epidermis surface. After incubation, skin surface was washed with 1 ml of distilled water and the alcoholic minoxidil solution was placed on the skin samples. The *in vitro* study was carried out for 8 h as described above.

2.8. Statistical analysis of data

Data analysis was carried out with the software package Microsoft Excel, version 2003. Results are expressed as mean ± standard deviation. One way analysis of variance (ANOVA) was used to substantiate statistical differences between groups, while Student's t-test was used for comparison between two samples. We used $p < 0.05$ as a minimal level of significance.

3. Results and discussion

3.1. Vesicle formation and characterization

During this work new types of liposomes were prepared by using different penetration enhancer molecules with soy lecithin and cholesterol as the main components of the vesicular bilayer. The new vesicular systems were prepared and characterized in order to evaluate the capability of the permeation enhancers to destabilize the lipid bilayers and/or to increase vesicle deformability and to improve cutaneous delivery of minoxidil. To this purpose labrasol, transcutol, and cineole were used in the composition of the new vesicular systems.

Labrasol is a safe, non-ionic hydrophilic surfactant (HLB = 14) developed by Gattefossé Corp. (Saint-Priest, France). It is a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethyleneglycol, with caprylic and capric acids being the predominant fatty acids (Koga et al., 2006).

Transcutol (diethylene glycol monoethylether) is a well-known non-toxic, biocompatible with skin, penetration enhancer. It is known to act by swelling of stratum corneum intercellular lipids without alteration of their multiple bilayer structure (Panachagnula and Ritschel, 1991; Godwin et al., 2002).

Cineole is a terpene. Terpenes are non-toxic and non-irritant to skin and are extensively used as permeation enhancers with both hydrophilic and lipophilic drugs. When skin is treated with terpenes, the existing network of hydrogen bonds between ceramides may get loosened and break since terpenes enter into the lipid

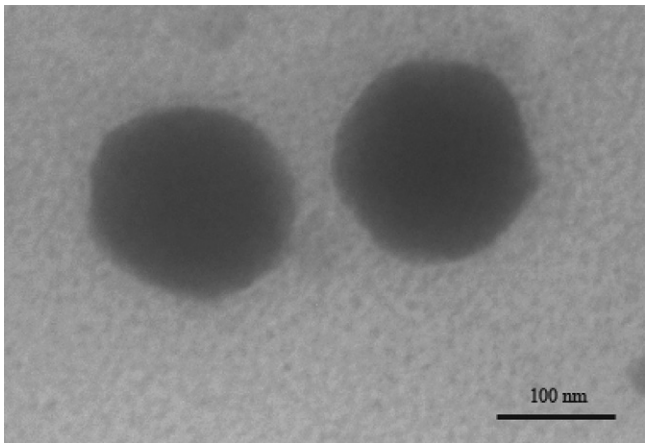

Fig. 1. Negative stain electron micrograph of minoxidil-loaded PEV-3 (cineole).

bilayer of SC (Jain et al., 2002). Very recently, invasomes prepared using 1% cineole were described as carriers for skin deposition of temoporfin (Dragicevic-Curic et al., 2009).

Compositions of the prepared vesicles are reported in Table 1. As a control, minoxidil incorporating traditional liposomes made with SL and DCP were used since this formulation had already shown to improve minoxidil skin deposition (Mura et al., 2007). PEVs were prepared using same composition of the control together with a proper amount of each permeation enhancer. Vesicle formation in the presence of the PE was confirmed by TEM and optical microscopy. In particular, TEM showed dark vesicular structures, as can be seen in Fig. 1, where it is possible to notice the outermost bilayer. Dark vesicles are probably due to strong interactions between the PE and phosphotungstic acid, which is able to selectively deposit electrons in the sample and enhance structural details.

PEVs were characterized by photon correlation spectroscopy (PCS) for size distribution. Fig. 2 shows mean size of the prepared vesicles 1–3 and control before and after lyophilization, and after purification procedure by exhaustive dialysis. PEVs mean size ranged between 140 and 195 nm and all suspension showed to be homogeneous and monodisperse with a polydispersity index (PI) < 0.3. In particular, PEVs prepared with cineole (PEV-3) showed the same average diameter of the control while PEV-1 and PEV-2, prepared with the hydrophilic PEs labrasol and transcutol, were larger. This is because hydrophilic molecules increase vesicle surface energy, which causes vesicle enlargement (Manconi et al., 2006).

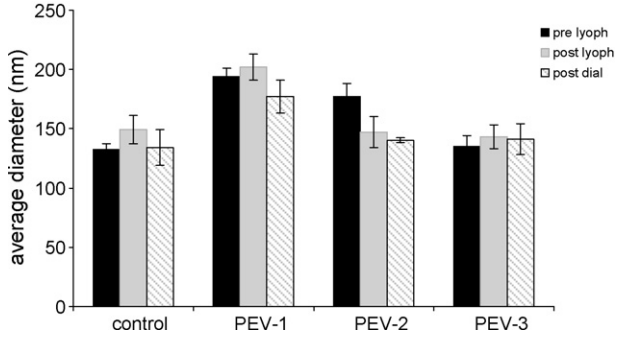

Fig. 2. *z*-Average diameter (nm) of minoxidil-loaded soy lecithin liposomes (control) and PEVs prepared by hydration–rehydration method: 1 day after production (pre-lyophilization), after freeze-drying (post-lyophilization) and after purification procedure (post-dyalisis). Error bars represent standard deviation ($n = 6$).

Table 2
Characterization of minoxidil vesicular suspensions prepared by hydration–rehydration method: minoxidil concentration, entrapment efficiency (EE), zeta potential (ξ), and solubility of minoxidil in each pure penetration enhancer (Cs).

Formulation	Minoxidil conc. (mg/ml)	EE (%)	ξ (mV)	Cs (mg/ml)
Control	32.55 ± 1.6	58.1 ± 1.2	−55 ± 2	2.2[a]
PEV-1 (labrasol)	38.40 ± 1.9	60.7 ± 3.0	−58 ± 2	17.01
PEV-2 (transcutol)	30.75 ± 1.5	59.2 ± 2.9	−52 ± 2	8.67
PEV-3 (cineole)	36.50 ± 1.8	71.3 ± 3.5	−57 ± 1	0.94

Each data is the mean ± standard deviation of at least three experimental determinations.
[a] Solubility in water.

Table 3
Determination of deformation index (DI) of vesicular suspensions.

Formulation	*z*-Diameter(nm) before extrusion	*z*-Diameter (nm) after extrusion	DI
Control	144 ± 3	127 ± 8	69
PEV-1 (labrasol)	202 ± 3	207 ± 16	400
PEV-2 (transcutol)	171 ± 6	177 ± 6	152
PEV-3 (cineole)	144 ± 3	140 ± 10	331

Each data is the mean ± standard deviation of at least six experimental determinations.

Formulations showed a good stability during vesicle reconstitution after freeze-drying, although this procedure was carried out without any cryoprotectant. The purification procedure did not affect vesicle size appreciably. These results demonstrate that the presence of the PE in the vesicular formulations did not affect vesicle stability. Moreover, Table 2 shows that zeta potential values of all formulations were very similar and highly negative (from −52 to −58 mV) ensuring prevention of aggregation process.

All PEV formulations were able to incorporate a good amount of minoxidil. In fact, as shown in Table 2, *E*% ranged from 59 (PEV-2, transcutol) to 71% (PEV-3, cineole). In particular, labrasol- and transcutol-containing formulations gave *E*% statistically similar to that of the control (≅60%). On the contrary, when the lipophilic PE cineol was used the highest *E*% (71%) was obtained. In order to clarify these results, minoxidil solubility in each pure PE was studied. As can be seen by Table 3, no correlation can be found between minoxidil solubility in the different PE and obtained *E*%. The highest drug solubility was obtained with the two hydrophilic PE that gave *E*% close to that of control. Minoxidil solubility in the lipophilic cineole was very low (0.94 mg/ml) but cineole-containing vesicles showed the highest loading capability. Therefore, the high *E*% obtained with the lipophilic terpene cineole is probably due to the particular molecular structure of this compound that can lead to a higher incorporation of minoxidil. It was reported that terpenes (cineole) act as PE by disrupting hydrogen bonds that link ceramides in the skin since alcoholic –OH is more electronegative than NH of amide I (Jain et al., 2002). The presence of cineole in the liposomal bilayer could have produced the same effect leading to an increased space for the minoxidil incorporation. A deeper study regarding interaction of cineole with liposomal bilayers is in progress by using FT-IR and DSC and results will be the object of a further paper.

The main feature of deformable vesicles in comparison with traditional liposomes is the elasticity of the vesicular membrane that is able to squeeze through pores smaller than their size. Deformability depends on the ability of the edge activator to destabilize phospholipids packing in liposomal bilayer and it is supposed to facilitate their capability to carry drugs into and through the skin layers (Cevc et al., 2002). Therefore, deformability of the prepared PEVs was studied by extruding them through polycarbonate membranes with pores smaller than their mean size. In Table 3, the

Table 4
Determination of minoxidil deposition into pig skin treated either with PEV suspensions or control.

Formulation	Dose minoxidil retained (%)			
	SC	Epidermis	Dermis	Total minoxidil delivered
Control	4.02 ± 0.15	2.33 ± 0.04	0.08 ± 0.01	6
PEV-1 (labrasol)	11.07 ± 0.77	8.95 ± 1.07	0.02 ± 0.01	20
PEV-2 (transcutol)	4.99 ± 0.42*	2.82 ± 0.38*	0.06 ± 0.01	8
PEV-3 (cineole)	12.85 ± 1.07	4.04 ± 0.09	0.09 ± 0.02	17

Each data is the mean ± standard deviation of at least six experimental determinations.
* $p < 0.05$ in comparison with control.

mean diameter of both control and PEVs before and after extrusion through the microporous membrane with a pore diameter of 50 nm is reported. As it is shown, while control liposomes had a reduction of their average diameter, PEVs size was nearly the same after their passage through membrane pores smaller by a factor of 4 than initial vesicle size. Moreover, the passage of PEVs through the 50 nm pores was almost complete, while only a portion of the control dispersion could be extruded. Therefore, results of these experiments pointed out a higher deformability of PEVs than the conventional liposomes as shown by Table 3 where deformability index (DI) for the studied formulations is also reported. Elasticity of the studied PEVs was also supported by the fact that the PI of all the PEV dispersions did not change after the extrusion. Thus, all the PEs used in formulation of the PEVs were able to give vesicles more deformable than the conventional liposomes although their different structure and physicochemical properties. As can be seen from the table, deformability increased in the following order: Control < PEV-2 (transcutol) < PEV-3 (cineole) < PEV-1 (labrasol).

Best results in terms of deformability were obtained by the hydrophilic labrasol (formulation 1), which was about 6 times more deformable than the control. On the contrary, as can be seen, DI of formulation 2, containing Transcutol®, is only twofold higher than the classic liposomes. As can be seen by the table, a great difference in the deformability of the PEVs obtained with the two hydrophilic penetration enhancers was observed. This is the consequence of the different structure of labrasol and transcutol. In fact, labrasol is a mixture of amphipatic compounds (see above) capable of acting as "edge activator" thus destabilizing the lipid bilayer and increasing its deformability. On the other hand, transcutol (diethylene glycol monoethylether) is a hydromiscible solvent that in low concentrations, as those used in this study, is mostly dispersed in the aqueous phase, where it is able to interact only with the polar heads of phospholipids.

As known, molecules show different affinity for the curved surfaces, the more hydrophilic tends to accumulate in the more strongly curved region, while the more hydrophobic in the membrane region with small curvature (Cevc et al., 1995) Thus, a different molecular structure can lead to a different distribution of the PE. The simultaneous presence in the membrane of different stabilizing (phospholipids) and destabilizing (enhancers) molecules and their tendency to redistribute in the non-uniformly stressed bilayers (Mishra et al., 2007) enable these liposomes to be much more elastic than the standard liposomes, and thus potentially well suited for skin penetration.

3.2. In vitro skin permeation studies

(Trans)dermal delivery of minoxidil through and into newborn pig skin was evaluated *in vitro* under non-occlusive conditions using vertical diffusion cells. No permeation of minoxidil through the whole skin thickness was detected in the present study using both control and PEV vesicles but all PEVs were able to improve skin deposition when compared to the classic liposomes. Minoxidil content in skin layers is reported in Table 4. Skin permeability values from all the systems are expressed as a percentage of accumulated drug in comparison with drug applied onto the skin.

As can be seen, the highest drug accumulation was found in the SC where PEV-1 (labrasol) and PEV-3 (cineole) enhanced minoxidil deposition by a factor of about three in comparison with the control ($p < 0.01$) while PEV-2 (transcutol) slightly improved drug deposition in comparison with the soy lecithin liposomes (by a factor of 1.3; $p < 0.05$). Therefore, the tested PEVs showed to be a better carrier for minoxidil than classic liposomes that are known not to be able to enter the SC. Using PEVs, drug accumulation in epidermis was also improved while accumulation in dermis was quite low (0.02–0.09%). The lowest enhancement in total skin strata was obtained with PEV-2 (transcutol), which had shown the lowest DI value. On the contrary, the highest deposition was obtained with PEV-1, which was the most elastic vesicular formulation.

Therefore, results obtained in this work show that drug delivery to the skin in strictly correlate to vesicle deformability, the higher the DI the higher the drug deposition in the skin. Consequently, these results allow us to hypothesize that these new vesicles are able to produce a depot in the skin layers from which minoxidil can be slowly released. Different outcomes obtained by the different tested liposomes also highlight that minoxidil delivery is dependent on the vesicle composition and these new deformable vesicles, PEVs, are able to improve *in vitro* minoxidil deposition in the outermost skin strata while reducing its systemic absorption.

In order to ascertain the role of the PE in the drug cutaneous delivery of minoxidil by using PEVs, permeation experiments were also carried out by comparing (trans)dermal delivery of minoxidil from PEVs with those obtained with solutions of "free" PE. Since the aim of this work was to find new and better delivery systems for minoxidil topical application, we choose the commercial solu-

Table 5
Results of *in vitro* permeation study from commercial solution of minoxidil (control), penetration enhancer-containing drug ethanolic solutions: percentage of minoxidil accumulated into and delivered through the pig skin at the end of the experiments (8 h) and total minoxidil delivered to and through the skin.

Formulations	Dose minoxidil delivered (%)				
	SC	Epidermis	Dermis	Receptor fluid	Total minoxidil delivered
Control	3.52 ± 0.32	9.31 ± 0.46	0.20 ± 0.06	0.16 ± 0.03	13
Labrasol solution	5.65 ± 0.87	6.47 ± 1.03	0.22 ± 0.06	1.15 ± 0.44	13
Transcutol solution	3.90 ± 0.82	4.48 ± 0.55	0.21 ± 0.03	1.95 ± 0.24	10
Cineole solution	4.41 ± 0.63	3.64 ± 0.82	0.43 ± 0.04	0.54 ± 0.09	9

Each data is the mean ± standard deviation of at least six experimental determinations.

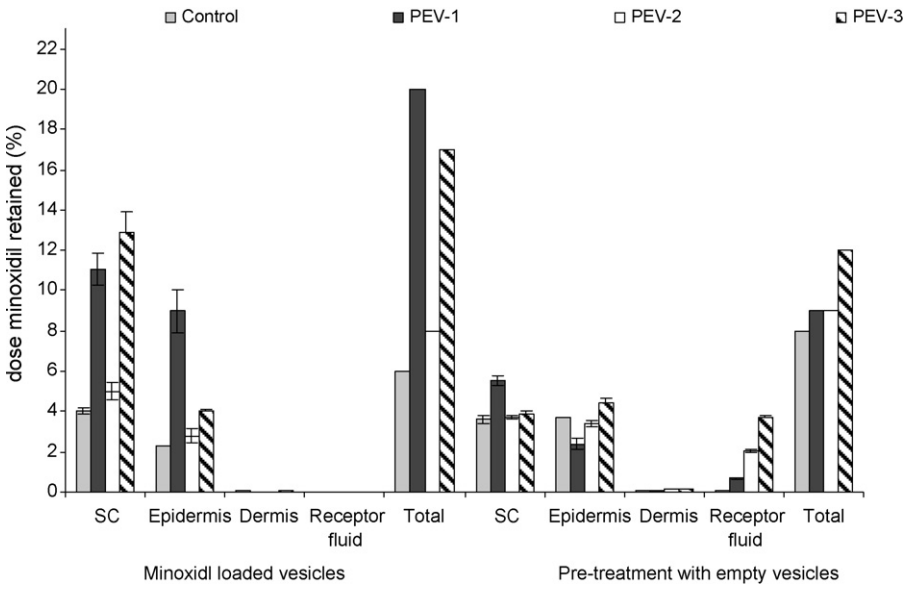

Fig. 3. Comparison of cutaneous and transdermal delivery of minoxidil after 8 h non-occlusive treatment with drug loaded PEVs and control and pre-treatment with corresponding empty vesicles.

tion of minoxidil as a control and as a base solution for studying the influence of each "free" PE. Penetration enhancers were used in the same amount employed in PEV formulations. Results are shown in Table 5 where it can be seen that when drug hydroalcoholic PE solutions were used, permeation of minoxidil through the newborn pig skin layers always occurred. Moreover, in comparison with the control, all PE solutions showed an improved drug permeation that was particularly evident when labrasol and transcutol were employed. This is probably due to the synergic action of the PE and the large amount of ethanol that is known to give a rapid fluidification of the SC lipids (Williams and Barry, 2007), with a reduction of the barrier function of the skin. On the contrary, PE solutions led to a decreased drug accumulation in the SC and only cineole solution was able to increase drug deposition in the dermis.

Comparison of results from *in vitro* skin treatment with PEVs and PE-containing drug solutions put in evidence the different outcomes obtained when the skin was treated with "free" or liposomal incorporated PE. Indeed, the inclusion of the PEs in elastic vesicles always led to an improved drug deposition in the outermost skin layer while the free PE in solution promoted transdermal drug delivery. However, comparison of the data in Tables 4 and 5 shows that when the most deformable vesicles were used (PEV-1 and PEV-3) the total amount of minoxidil delivered to the skin was higher than what found into and through the skin with the corresponding PE solutions (labrasol and cineole). The contrary happened with the conventional liposomes and transcutol-containing PEV-2. Therefore, formulations containing different PE leads to different results. This also indicates that mechanism by which PEVs interacts with the skin could not be only a penetration enhancing effect.

Several studies have been investigating the possible mechanisms by which deformable vesicles can act as skin drug delivery systems. Although they have not fully understood yet, two main processes have been suggested: vesicles can act as drug carriers or vesicles can act as penetration enhancers.

According to the first mechanism, intact vesicles enter the SC carrying their content while in the second one vesicle components enter the SC modifying the intercellular lipid bilayers thus improving pathway for free drug molecules into and through the SC. Most recent studies strongly support the first hypothesis suggesting that elastic vesicles can penetrate intact the skin to a certain extent thus acting as drug carriers (El Maghraby et al., 1999, 2000; Cevc et al., 2002; Honeywell-Nguyen and Bouwstra, 2003). This mechanism was first suggested by Cevc and Blume (1992) and Cevc et al. (2002) who proposed that the elastic vesicles (Transfersome®) can penetrate intact the skin spontaneously under the influence of the naturally occurring in vivo transcutaneous hydration gradient even reaching the blood circulation. However, Bouwstra and Honeywell-Nguyen (2002) noticed that even in fully hydrated state, the water content in the lowest SC layers close to the viable epidermis is much lower than in central regions of SC. Thus, they suggested that, as a result of the osmotic force, vesicles will not penetrate beyond the level of the lowest SC layers and drugs have to be released from vesicles in order to reach the systemic circulation (Bouwstra and Honeywell-Nguyen, 2002; Honeywell-Nguyen et al., 2006).

As written above, results of studies showing that deformable vesicles are only able to improve skin deposition of some drugs were already reported (El Maghraby et al., 2001; Trotta et al., 2002; Elsayed et al., 2007) and could support this proposal. However, several studies also support the possible penetration enhancing mechanism for the deformable vesicles (Verma et al., 2003; Elsayed et al., 2006).

Therefore, in the present research, to shed some lights on the possible mechanism of skin drug delivery from PEVs, the influence of skin pre-treatment with empty PEVs and conventional liposomes (control) was also studied. To this purpose pig skin was first treated with empty vesicles and then the commercial minoxidil solution was applied. Table 6 reports results of the minoxidil delivery to and through the skin after the pre-treatment experiments. Comparison of these results with those of Table 4 shows that the pre-treatment generally reduced drug accumulation in the SC but improved deposition in dermis as well as transdermal delivery with respect to minoxidil loading PEVs (Fig. 3). According to literature, these results should be interpreted as a prove of the enhancing properties of the deformable vesicles (Kirjavainen et al., 1996). However, these results could also be the consequence of the presence of ethanol in the commercial minoxidil formulation (Williams and Barry, 2007). Moreover, although the pre-treatment with empty PEVs led to an improved drug transport through the skin, the total amount of drug delivered to and through the skin was lower in the case of the highly deformable vesicles, formulations 1 (labrasol) and 3 (cineole), as can also shown in Fig. 3. Indeed, the total drug amount delivered by PEV-1 and PEV-3 were, respectively, 20% and 17% of the applied

Table 6
Results of *in vitro* permeation study after pre-treatment of pig skin with empty PEVs and conventional liposomes (control) followed by treatment with the commercial solution of minoxidil (5%): percentage of minoxidil accumulated into and delivered through the skin at the end of the experiments (8 h) and total minoxidil delivered to and through the skin.

Formulations	Dose minoxidil delivered (%)				
	SC	E	D	Receptor fluid	Total minoxidil delivered
Control	3.64 ± 0.21	3.75 ± 0.22	0.10 ± 0.01	0.10 ± 0.01	8
PEV-1 (labrasol)	5.48 ± 0.24	2.39 ± 0.25	0.06 ± 0.01	0.69 ± 0.05	9
PEV-2 (transcutol)	3.72 ± 0.07	3.45 ± 0.19	0.13 ± 0.01	2.04 ± 0.10	9
PEV-3 (cineole)	3.89 ± 0.16	4.42 ± 0.29	0.16 ± 0.02	3.75 ± 0.03	12

Each data is the mean ± standard deviation of at least six experimental determinations.

drug, while after the pre-treatment the total amount of recovered drug decreased to 9 (PEV-1) and 12% (PEV-3). On the contrary, the control and the transcutol-containing PEV-2 showed a reduced total drug skin delivery than with the pre-treatment.

Therefore, these results further support our suggestion that the mechanism by which deformable vesicle can act is strongly affected by the vesicle deformability that in turn is affected by the vesicle composition. In particular, the finding that the highest deformable formulations 1 and 3 were able to deliver to the skin a higher drug amount than what obtained by the pre-treatment lead us to hypothesize that in this case the intact vesicle penetration could be the main mechanism: PEV-1 and PEV-3 may penetrate intact the skin by entering the SC where they form a depot from which the drug is slowly released.

However, further studies using different composition are necessary to prove this suggestion. Our study is still in progress and we are now evaluating the skin delivery by PEVs using confocal laser microscopy, FT-IR, and other model drugs.

4. Conclusions

In this study, PEVs, vesicles containing different penetration enhancers, have shown a good ability to increase minoxidil accumulation in the upper skin layers without any transdermal delivery thus improving cutaneous drug bioavailability. Moreover, the finding that the most deformable PEVs were able to deliver a higher total drug amount to the skin in comparison with traditional liposomes, PE-containing ethanolic solution of the drug as well as after pre-treatment with empty vesicles leads us to conclude that PEVs can be a potential innovative carriers for improving topical delivery of minoxidil.

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