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Nanocarriers for topical administration of resveratrol: A comparative study

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ABSTRACT

The *trans*-resveratrol (*t*-res), a non-flavonoid polyphenol extracted from different plants, has recently earned interest for application on the skin for different applications. In this work, the potential of nanocarriers, namely transfersomes and ethanol-containing vesicles, to deliver *t*-res into/through the skin was investigated. Thus, transfersomes with different surfactants, namely polysorbate 80 (Tw80), sodium cholate (SC) and sodium deossicholate (SDC) and ethanol-containing vesicles with different lipid composition, namely soy phosphatidylcholine (SPC) and cholesterol (chol), encapsulating *t*-res were prepared and characterized. The nanocarriers had a mean diameter ranging between 83 and 116 nm with a high *t*-res encapsulation efficiency (≥70%). Moreover, cytotoxicity as well as the inhibition of production of reactive oxygen species (ROS) and lipid peroxidation, following incubation of H_2O_2-stimulated human keratinocyte (HaCaT) with *t*-res, as free or encapsulated into the nanocarriers, were investigated. Only blank nanocarriers containing Tw80 or ethanol were cytotoxic and led to increase of ROS, but this effect was not observed when using nanocarriers encapsulating *t*-res. Finally, permeation studies on porcine skin carried out on Franz diffusion cells, showed that only ethanol-containing vesicles based SPC were able to promote *t*-res permeation through the skin.

1. Introduction

The resveratrol (3,5,4′-trihydroidroxystilbene) is a non flavonoid polyphenol, present in different plants. It is extracted from the grapes peel of the common red grapewine (*Vitis vinifera*), and exists in two different isoforms: the *trans* and the *cis*-resveratrol. Several beneficial effects have been attributed to resveratrol and its use has been proposed in the treatment or prevention of different diseases (Baur and Sinclair, 2006). Special attention has been devoted to the topical application of resveratrol in different physiological and pathological conditions. In particular, resveratrol has been used to prevent skin cancer (Jang et al., 1997; Bhat and Pezzuto, 2002) or to treat inflammatory diseases such as psoriasis (Hrenn et al., 2006). Moreover, the use of resveratrol as antimicrobial and antiviral agent has also been proposed to treat human skin infection (Chan, 2002; Docherty et al., 2005). Finally, it has been reported the positive role of resveratrol in wound healing processes (Khanna et al., 2002). Recently, our group demonstrated clinically relevant and statistically significant decrease of symptoms related to acne *vulgaris* by using a resveratrol-containing gel (Fabbrocini et al., 2011). This effect has been explained by resveratrol interference with the hyperproliferation of keratinocytes, the inflammatory response, and P. acnes growth to be exerted at the level of the follicular epithelium, located into the dermis. From here, there is a great interest to increase resveratrol concentration into the dermis. However, the skin permeation/accumulation of resveratrol can be significantly affected by the formulation (Hung et al., 2008). Moreover, resveratrol is also a very unstable molecule and, following exposure to solar rays, it converts its *trans* form in the less active *cis* form (Orallo, 2006). Thus, the topical use of the *trans*-resveratrol for cosmetic or therapeutic purposes requires the development of suitable formulations, able to assure bioavailability and stability of the active. The use of nanotechnology for topical administration of drugs has showed several advantages and a number of products are today on the market or in clinical trials (Elsayed et al., 2007a,b). Different strategies have been investigated to modulate vesicle composition in order to improve

Abbreviations: *t*-res, *trans*-resveratrol; Tw80, polysorbate 80; SC, sodium cholate; SDC, sodium deossicholate; SPC, phosphatidylcholine; chol, cholesterol; ROS, reactive oxygen species; HaCaT, human keratinocyte; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HPLC, high performance liquid chromatographic; RP-HPLC, reverse-phase chromatography; H_2DCFDA, 2′,7′-dichlorofluorescin-diacetate.

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the ability of nanocarriers to carry drugs and macromolecules to deeper tissues, leading to two novel vesicular carriers, ultraflexible lipid-based elastic vesicles (transfersomes) and ethosomes, that gave the most promising results. In particular, transfersomes are elastic vesicles, which were first introduced in the early 1990s by Cevc and Blume (1992). They have been reported to be able to penetrate intact skin, carrying therapeutic concentrations of drugs when applied under non-occlusive conditions (Cevc, 1996; El Maghraby et al., 2000a,b; Cevc and Blume, 2001; Trotta et al., 2004; Honeywell-Nguyen et al., 2004). They are liquid-state vesicles with a highly deformable membrane (Cevc and Gebauer, 2003) which permits their easy penetration through skin pores much smaller than the vesicles' size. They have been proven to be superior to conventional gel-state and even liquid-state vesicles in terms of both, the enhancement of drug permeation and interactions with human skin (van den Bergh et al., 2001; Dragicevic-Curic et al., 2010). Vesicles composed of phospholipids, water and ethanol have been named ethosomes (Touitou et al., 2000; Elsayed et al., 2007a). Several studies addressed the effect of ethanol on physicochemical characteristics of the ethosomal vesicles (Dayan and Touitou, 2000; López-Pinto et al., 2005; Elsayed et al., 2007a). Ethosomes were reported to be effective at delivering molecules to and through the skin to the systemic circulation. Contrary to deformable liposomes, ethosomes were able to improve skin delivery of drugs both under occlusive (Dayan and Touitou, 2000; Ainbinder and Touitou, 2005; López-Pinto et al., 2005; Paolino et al., 2005) and non-occlusive conditions (Dayan and Touitou, 2000; Elsayed et al., 2007a). The exact process of drug delivery by ethosomes is still unknown. However, a synergistic mechanism between ethanol, vesicles and skin lipids can be suggested (Touitou et al., 2000). Indeed, ethanol could interact with lipid molecules in the polar headgroup region, resulting in an increasing of the stratum corneum fluidity. In addition, the ethosome itself may interact with the stratum corneum barrier. Ethanol may also provide the vesicles with soft flexible characteristics which allow them to more easily penetrate into deeper layers of the skin.

In this study, we prepared and characterized different vesicles for topical application of *t*-res. In particular, we prepared transfersomes and ethanol-containing vesicles encapsulating *t*-res. We also investigated the effects of *t*-res, alone or encapsulated into the nanocarriers, on the generation ROS and lipid peroxidation, in H_2O_2-stimulated human keratinocytes. Finally, the *t*-res accumulation into dermis and permeation through skin, following incubation with the different nanocarriers, was evaluated on porcine skin.

2. Materials and methods

2.1. Materials

t-res, chol, Tw80, SC, SDC, Sephadex G-50150, N,N-dimethylformamide, 2′,7′-dichlorofluorescin-diacetate (H_2DCFDA) and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (USA). Methanol (HPLC grade), ethanol, chloroform, glacial acetic acid were provided by Carlo Erba Reagenti (Italy). Soy phosphatidylcholine (SPC) was kindly offered from Lipoid GmbH (Cam, Switzerland). Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were from Biowhittaker, fetal bovine serum (FBS) was from Lonza BioWhittaker (Milan, Italy).

2.2. Preparation of transfersomes and ethanol-containing vesicles

The transfersomes and ethanol-containing vesicles have been prepared by the thin lipid film hydration method. Briefly, for the transfersomes, mixture composed of SPC/Tw80 (9:1 molar ratio) and SPC/SC or SPC/SDC (9:1 weight ratio), containing *t*-res (0.1 mg/ml), was dissolved in a mixture of chloroform/methanol (2:1 v/v). For the ethanol-containing vesicles, the lipid mixture composed of SPC or SPC/chol (2:1 weight ratio), containing *t*-res, was dissolved in a mixture of chloroform/methanol (2:1 v/v). The organic solution was added to a 50 ml round-bottom flask for each preparation, and the solvent was removed under reduced pressure by a rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) under nitrogen atmosphere. The resulting film was then hydrated (40 mg PC/ml hydration solution) with phosphate buffer solution (PBS pH 7.2) or an aqueous solution containing 40% (v/v) of ethanol, in the case of tranfersomes and ethanol-containing vesicles, respectively, followed by agitation on vortex in presence of glass beads. All formulations, with the exception of transfersomes containing Tw80, were then extruded using a thermobarrel extruder system (Northern Lipids Inc., Vancouver, BC, Canada) passing the suspension under nitrogen through polycarbonate membrane (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK) with decreasing pore size (from 0.4 to 0.1 μm). In the case of transfersomes containing Tw80, suspensions were sonicated by micro-tip (Sonicator 3000, Misonix, Farmingdale, USA) for 15 min with intervals of 30 s each 60 s. The unencapsulated *t*-res was removed by passing the nanocarrier suspension through Sephadex G-50150 column and eluted in aqueous or PBS (pH 7.2) solution. All the steps of the preparation were carried out avoiding exposure of *t*-res to the light. The suspensions were then stored at 4 °C in the dark. Each formulation was prepared in triplicate.

2.3. Transfersome and ethosome size and zeta potential

The mean diameter of the transfersomes and ethanol-containing vesicles, soon after preparation and after storage at 4 °C, was determined at 20 °C by photon correlation spectroscopy (N5, Beckman Coulter, Miami, USA). Each sample was diluted in deionizer/filtered (0.22 μm pore size, polycarbonate filters, MF-Millipore, Microglass Heim, Italy) water and analyzed with detector at 90° angle. As measure of the particle size distribution, polydispersity index (P.I.) was used.

The zeta potential (ZP) of the transfersomes and ethanol-containing vesicles was determined in distilled water at 20 °C by Zetasizer Nano Z (Malvern, UK). For each batch, mean diameter, size distribution and ZP were the mean of three measures. For each formulation, the mean diameter and P.I. were calculated as the mean of three different batches ($n = 3$).

2.4. t-res encapsulation and stability into transfersomes and ethanol-containing vesicles

t-res dosage was carried out by a high performance liquid chromatographic (HPLC) method (Chen et al., 2007). The HPLC system consisted of an isocratic pump (LC-10A VP, Shimadzu, Kyoto, Japan) equipped with a 7725i injection valve (Rheodyne, Cotati, USA), SPV-10A UV–vis detector (Shimadzu) set at the wavelength of 303 nm. The system was controlled by a SCL-10A VP System Controller (Shimadzu) connected with a computer. Chromatograms were acquired and analyzed by a Class VP Client/Server 7.2.1 program (Shimadzu). The quantitative analysis of *t*-res was performed by reverse-phase chromatography (RP-HPLC) on a Luna 5 μm C_{18} column 250 mm × 4.60 mm, 110 Å (Phenomenex, Klwid, USA) equipped with a security guard. The mobile phase was a mixture 50:50 (v/v) of water and methanol containing the 0.5% (v/v) of acetic acid. The *t*-res analysis was performed in isocratic condition, at a flow rate of the mobile phase of 1 ml/min and at room temperature. HPLC analysis was also used to investigate the conversion of *t*-res in its *cis* isomer. The *cis* isomer of resveratrol was prepared as

reported elsewhere (Fan et al., 2008). Briefly, a *t*-res solution was exposed at the sun light for 5 days at room temperature. The HPLC analysis of the resulting solution showed two chromatographic peaks: according with the literature, one was attributed to *t*-res and the other, with higher retention time, attributed to the *cis* isomer of resveratrol.

To determine the amount of encapsulated *t*-res, 100 μl of transfersomes or ethanol-containing vesicles suspension was added to 900 μl of methanol; the resulting solution was centrifuged at 13,000 rpm for 30 min and the supernatant was analyzed by RP-HPLC. *t*-res loading into transfersomes or ethanol-containing vesicles was investigated soon after preparation and after storage at 4 °C. For each batch, *t*-res loading was the mean of three measures. For each formulation, the *t*-res loading was calculated as the mean of the measures obtained in three different batches ($n = 3$). The amount of *t*-res loaded into the nanocarriers was expressed as *t*-res actual loading and encapsulation efficiency, calculated as μg of *t*-res/mg of SPC and percent ratio between *t*-res actually loaded into transfersomes or ethanol-containing vesicles and *t*-res theoretical loading, respectively. The phospholipid content of the carrier suspension was determined by the Stewart assay (Stewart, 1980). Briefly, an aliquot of the liposome suspension was added to a two-phase system, consisting of an aqueous ammonium ferrithiocyanate solution (0.1 N) and chloroform. The concentration of SPC was obtained by measure of the absorbance at 485 nm into the organic layer.

2.5. Cell culture

The human keratinocyte HaCaT cell line was cultured at 37 °C in humidified 5%CO_2/95% air in DMEM containing 10% fetal bovine serum 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were incubated with transfersomes or ethanol-containing vesicles, blank or containing *t*-res (0.5, 1.0 and 2.0 μg/ml), or with *t*-res (2.0 μg/ml) in the presence or absence of H_2O_2 (10%). Transfersomes or ethanol-containing vesicles (final *t*-res concentration in the cell medium 0.5, 1.0 and 2.0 μg/ml) or naked *t*-res (2.0 μg/ml) were added to the cells 10 min before H_2O_2 (10%) challenge.

2.6. Cell viability

The cell viability was determined by using MTT conversion assay (De Stefano et al., 2007). Briefly, 100 μl MTT (5 mg/ml in complete DMEM) were added and the cells were incubated for an additional 3 h. After this time point the cells were lysed and the dark blue crystals solubilized with 500 μl of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620 nm filter. The cell viability in response to treatment with test compounds was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

2.7. Measurement of reactive oxygen species

The formation of reactive oxygen species (ROS) was evaluated by means of the probe H_2DCFDA as described elsewhere (De Stefano et al., 2007).

Briefly, HaCaT cells were grown in DMEM containing 10% (v/v) fetal bovine serum, then were plated at a density of 1.5×10^4 cells/well and then incubated in the growth medium containing 5 μM H_2DCF-DA for 2 h at 37 °C. H_2DCF-DA is a nonfluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular reactive oxygen species, H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were washed twice with PBS buffer; thereafter, the medium was replaced with fresh medium and cells were incubated with transfersomes or ethanol-containing vesicles, blank or containing *t*-res (0.5, 1.0 and 2.0 μg/ml) or naked *t*-res (2.0 μg/ml) alone in the presence of H_2O_2 (10%). After treatment, cells were washed twice with PBS buffer and plates were placed in a fluorescent microplate reader (Perkin Elmer LS55 Luminescence Spectrometer; Perkin Elmer, Beaconsfield, UK). Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each experiment, fluorescence increase was measured in ten replicate cultures ($n = 10$) for each treatment.

2.8. Malondialdehyde assay

Lipid peroxidation products from HaCat cells were measured by the thiobarbituric acid colorimetric assay. Briefly, cells were stimulated with H_2O_2 after incubation with the transfersomes or ethanol-containing vesicles formulations in the absence or presence or resveratrol. Then, cells were washed three times with PBS 1× and scraped in PBS 1× containing 0.5 mM EDTA and 1.13 mM butylated hydroxytoluene. Cell lysis was performed by means of six cycles of freezing and thawing. One milliliter of 10% (w/v) trichloroacetic acid was added to 450 μl of cellular lysate. After centrifugation at 1500 × *g* for 10 min, 1.3 ml 0.5% (w/v) thiobarbituric acid was added, and the mixture was heated at 100 °C for 20 min. After cooling, malonyldialdehyde (MDA) formation was recorded (A530 nm and A550 nm) in a Perkin-Elmer LS-5B spectrofluorimeter. The results are presented as RFU.

2.9. Ex vivo experiments

Freshly excised skin from pig ear was mounted on the receptor compartment of a Franz diffusion cell assembly with the *stratum corneo* (SC) side facing upwards into the donor compartment. Eight milliliters of 3:7 (v/v) ethanol-pH 7.4 buffer was used as the receptor medium. The donor compartment was filled with 1 ml of a mix ethanol/water (1:1 v/v) in the case of free *t*-res or a suspension of the nanovectors in PBS. For all samples, the *t*-res concentration in the donor compartment was 0.1 mg/ml. The available diffusion area between compartments was 0.6 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals (0.5, 12, 4, 6, 24 h), 300 μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh medium. The duration of this experiment was 24 h. The *t*-res concentration in the receiver samples was determined by filtration of the solution and following HPLC analysis. The amount of *t*-res accumulated in the dermis was determined as previously reported (Detoni et al., 2012). Briefly, after 24 h the experiment was stopped, and the dermis was recovered by heating the skin with an hairdryer and a spatula. *t*-res was extracted from the dermis with a mix of acetonitrile/water (3:1 v/v) and 1 h of sonication in a sonicator bath (3510, Branson Ultrasonics, USA). The resulting solution was filtered and the *t*-res concentration was determined by HPLC. Finally, the dermis was dried under vacuum and weighted. *t*-res accumulation was expressed as ng of *t*-res/mg of dermis. All the experiments were carried out in triplicate.

2.10. Statistical analysis

Results are expressed as the means ± SEM of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *p*-value for multiple

Table 1
Composition of the different formulations.

Formulation	Lipid composition	Additives in the lipid bilayer and additive/lipid ratio	Hydration phase
Tw80-Trans	SPC	Tw80 (1/9 mol/mol)	PBS
SDC-Trans	SPC	SDC (1/9 w/w)	PBS
SC-Trans	SPC	SC (1/9 w/w)	PBS
SPC-Etho	SPC	–	Ethanol 40°
SPC/chol-Etho	SPC/chol (2:1 weight ratio)	–	Ethanol 40°

comparison test. The level of statistically significant difference was defined as $p < 0.05$.

3. Results

3.1. Characteristics of the transfersomes and ethanol-containing vesicles containing t-res

Different formulations based on transfersomes and ethanol-containing vesicles containing *t*-res were prepared. In particular, in the case of transfersomes, three different edge activators, namely Tw80, SC ad SDC, were used. In the case of ethanol-containing vesicles, vesicles based on different lipid composition, namely SPC or SPC/chol, were prepared. The composition of the different formulations is reported in Table 1. All the formulations were characterized in terms of size, ZP and *t*-res encapsulation, soon after preparation and upon storage at 4 °C. The results are reported in Tables 2 and 3. In the case of transfersomes, mean diameter, soon after the preparation, was about 81.5, 111.5 and 105.4 nm for Tw80-Trans, SDC-Trans and SC-Trans, respectively. The type of surfactant significantly affected transfersomes size distribution. Actually, transfersomes based on SC and SDC showed a P.I. lower than 0.15, while, when using Tw80, a P.I. higher than 0.4 was found. Size characteristics of transfersomes did not significantly changed during storage at 4 °C for 60 days. Tw80-Trans, SDC-Trans and SC-Trans showed a ZP of about −7, −13 and −22 mV, respectively, without any significant difference during the storage.

In the case of ethanol-containing vesicles, namely the formulations SPC-Etho and SPC/chol-Etho, the size characteristics measured soon after preparation were not affected by the lipid composition. In particular, a mean diameter between 92.5 and 94.0 nm and a P.I. about 0.2 were found for both formulations. However, size characteristics of ethanol-containing vesicles, significantly changed during storage at 4 °C, with a progressive increase of mean size, while P.I. remain unaffected. The presence of chol did not significantly improved ethosome physical stability. Finally, SPC-Etho and SPC/chol-Etho showed a ZP of about −9 and −13 mV, respectively, without significant differences following storage.

3.2. Encapsulation of t-res into transfersomes and ethanol-containing vesicles

In our experimental conditions, HPLC analysis of *t*-res resulted in a chromatographic peak at the retention time (RT) of about 5 min. In the same conditions, an aqueous solution of *t*-res previously exposed to sun light, resulted in an further chromatographic peak at the RT of about 7 min. According with literature (Fan et al., 2008), this second chromatographic peak was attributed to the *cis* isomer of resveratrol. In all cases, the HPLC analysis of *t*-res extracted by the nanocarriers showed a chromatographic peak at the RT of 5 min, while any peak was detected at the RT of *cis*-resveratrol (data not shown). The *t*-res actual loading of the different formulations is reported in Table 3. In the case of transfersomes, high encapsulation efficiencies were obtained. In particular, a *t*-res actual loading of about 2.5 μg/mg SPC in the case of Tw80-Trans and SDC-Trans (encapsulation efficiency about 100%) and about 2.4 μg/mg SPC in the case of SC-Trans (encapsulation efficiency about 95.6%) were found. Compared to transfersomes, ethosome-based formulations showed lower encapsulation efficiencies. In particular, ethanol-containing vesicles had an actual loading of about 1.8 μg/mg SPC (encapsulation efficiency about 70%), without significant difference between the two formulations. *t*-res actual loading gradually decreased during storage, with the highest *t*-res release in the case of Tw80-Trans.

3.3. Effect of transfersomes and ethanol-containing vesicles containing t-res on cell viability

Exposure of HaCaT to blank transfersomes (Fig. 1) or blank ethanol-containing vesicles (Fig. 2) for 24 h caused a general reduction of cell viability, as compared to untreated cells, with the higher toxicity observed in the case of the Tw80-containing formulation (Fig. 1A). In contrast, encapsulating *t*-res into the nanocarriers reverted cell viability in a concentration-dependent manner (Figs. 1 and 2). In particular, SDC-Trans or SC-Trans and SPC-Etho or SPC/chol-Etho did not affect cell viability (Figs. 1 and 2). Finally, *t*-res in free form, at the concentration of 2 μg/ml (the highest used with the carriers), was not cytotoxic (Figs. 1 and 2).

3.4. Effect of transfersomes and ethanol-containing vesicles containing t-res on intracellular ROS generation

Incubation of cells with blank Tw80-Trans, blank SDC-Trans or blank SC-Trans did not cause a significant increase of intracellular ROS, as compared to untreated cells (Fig. 3). Incubation of cells with H_2O_2 resulted in an increased ROS production, which was significantly inhibited, in a concentration-dependent manner, by Tw80-Trans (by 61.93 ± 0.16%, 33.94 ± 0.13% and 11.01 ± 0.21%, respectively; $n = 12$), SDC-Trans (by 48.17 ± 0.14%, 33.49 ± 0.11% and 22.01 ± 0.07%, respectively; $n = 12$) or SC-Trans (by 62.40 ± 0.20%, 50.91 ± 0.10% and 42.70 ± 0.19%, respectively; $n = 12$). In the case of ethanol-containing vesicles, the addition of blank SPC-Etho or blank SPC/chol-Etho caused an accumulation of ROS which was significantly reduced by SPC-Etho (by 7.42 ± 0.13%, 25.00 ± 0.09% and 34.37 ± 0.08%, respectively; $n = 12$) as well as SPC/chol-Etho (by 23.44 ± 0.13%, 36.33 ± 0.09% and 41.79 ± 0.09%, respectively; $n = 12$). *t*-res alone, at the highest concentration (2 μg/ml), significantly inhibited ROS generation (by 27.34 ± 0.19%; $n = 12$) (Figs. 3 and 4).

3.5. Effect of transfersomes and ethanol-containing vesicles containing t-res on lipid peroxidation

To explore whether trasferosomes or ethanol-containing vesicles were capable of preventing H_2O_2-induced lipid peroxidation, MDA assay was performed. Incubation of cells with H_2O_2 resulted in an increased generation of peroxidized lipids (Figs. 5 and 6). Incubation of cells with blank Tw80-Trans caused a significant increase of lipid peroxidation. This effect was reverted by using Tw80-Trans (by 46.76 ± 0.14%, $n = 6$). Cell incubation with SDC-Trans, blank or containing *t*-res, significantly reduced generation of peroxidized lipids (by 53.96 ± 0.08% for SDC-Trans; $n = 6$). In the

Table 2
Mean diameter, size distribution and zeta potential of transferosomes and ethanol-containing vesicles containing *t*-res, after preparation and following storage at 4 °C.

Formulations	Time (days)										
	Soon after preparation			7			30			60		
	Mean diameter (nm ± S.D.)	P.I. ± S.D.	ZP ± S.D.	Mean diameter (nm ± S.D.)	P.I. ± S.D.	ZP ± S.D.	Mean diameter (nm ± S.D.)	P.I. ± S.D.	ZP ± S.D.	Mean diameter (nm ± S.D.)	P.I. ± S.D.	ZP ± S.D.
Tw80-Trans	81.5 ± 2.0	0.4 ± 0.1	−7.1 ± 0.6	77.4 ± 0.5	0.3 ± 0.1	−7.1 ± 0.4	80.1 ± 1.3	0.3 ± 0.0	−7.1 ± 0.4	78.4 ± 1.8	0.3 ± 0.0	−7.1 ± 0.4
SDC-Trans	111.5 ± 8.4	0.1 ± 0.0	−12.2 ± 1.0	112.2 ± 8.7	0.1 ± 0.1	−12.3 ± 1.0	112.0 ± 8.4	0.1 ± 0.0	−11.6 ± 1.1	110.7 ± 3.7	0.1 ± 0.1	−11.5 ± 1.1
SC-Trans	105.4 ± 7.9	0.1 ± 0.0	−22.4 ± 0.2	106.0 ± 6.9	0.1 ± 0.0	−21.7 ± 0.4	106.2 ± 6.0	0.1 ± 0.0	−23.2 ± 0.5	105.5 ± 6.93	0.1 ± 0.0	−22.2 ± 0.7
SPC-Etho	92.5 ± 5.9	0.2 ± 0.0	−9.9 ± 1.1	95.2 ± 1.5	0.1 ± 0.1	−9.6 ± 1.1	96.5 ± 13.1	0.2 ± 0.1	−9.8 ± 1.1	91.6 ± 2.8	0.2 ± 0.0	−10.0 ± 1.0
SPC/chol-Etho	94.0 ± 2.5	0.2 ± 0.04	−13.5 ± 0.7	96.7 ± 4.8	0.2 ± 0.0	−12.9 ± 0.8	98.6 ± 10.7	0.2 ± 0.0	−12.8 ± 0.2	99.7 ± 8.8	0.2 ± 0.1	−13.0 ± 0.3

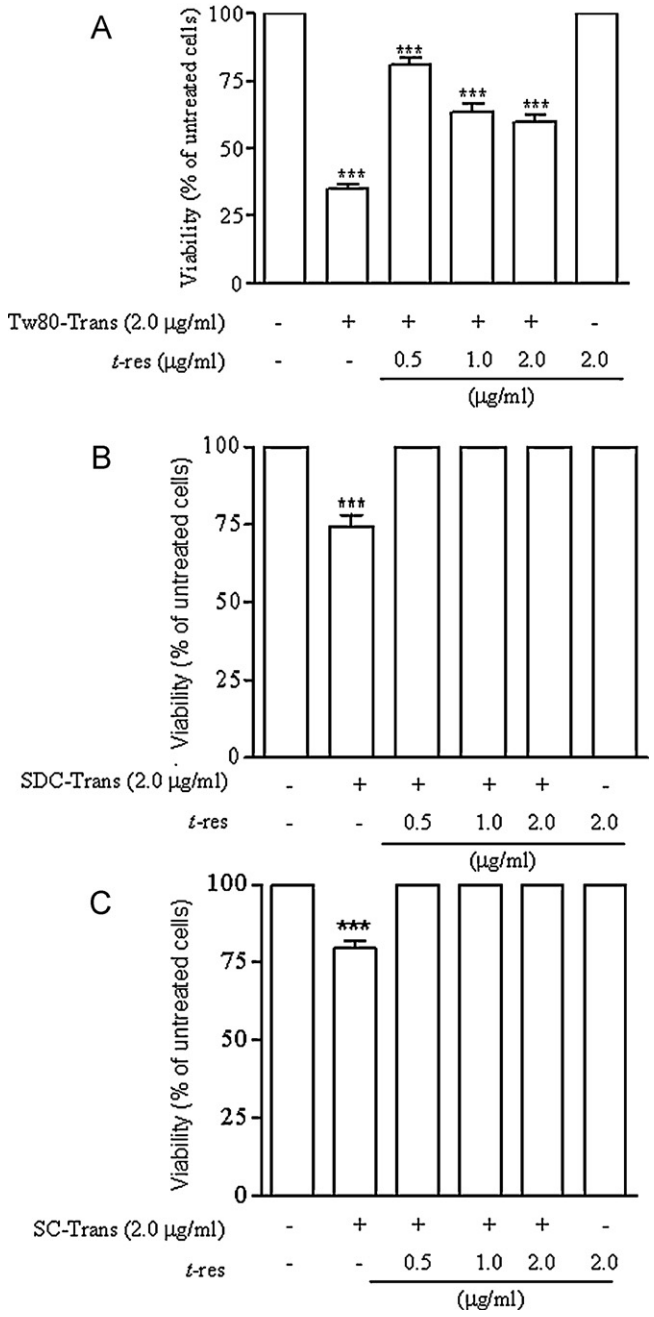

Fig. 1. MTT assay shows the effect of Tween80 (A), SDC (B) or SC (C) transferosomes in the absence or presence of *t*-res on cell viability. Data are mean ± S.E.M. of six experiments in triplicate. ***$p < 0.001$ *vs.* unstimulated cells.

case of SC-Trans, blank vesicles reduced lipid peroxidation, and this effect was increased when using *t*-res containing SC-Trans (by 41.73 ± 0.11%, $n = 6$). Finally, cell incubation with blank SPC-Etho or blank SPC/chol-Etho significantly inhibited lipid peroxidation. This effect was only slightly, but not significantly, increased by using SPC-Etho as well as SPC/chol-Etho (by 34.53 ± 0.12% and 36.69 ± 0.12%, respectively; $n = 6$) (Figs. 6 and 7).

3.6. Ex vivo experiments

The accumulation/permeation of *t*-res in/through the porcine skin were investigated on Franz cells. In the receptor compartment, free *t*-res or *t*-res-encapsulating nanocarriers, was used at the concentration of 0.1 mg/ml. The results are shown in Figs. 7 and 8.

Table 3
t-res actual loading of transferosomes and ethanol-containing vesicles, after preparation and following storage at 4 °C.

Formulations	Actual loading (μg *t*-res/mg SPC ± S.D.)			
	Soon after preparation	After 7 days of storage at 4 °C	After 30 days of storage at 4 °C	After 60 days of storage at 4 °C
Tw80-Trans	2.50 ± 0.32	2.39 ± 0.42	2.30 ± 0.57	1.36 ± 0.61
SDC-Trans	2.49 ± 0.30	2.35 ± 0.34	2.03 ± 0.23	1.96 ± 0.09
SC-Trans	2.39 ± 0.23	1.83 ± 0.35	1.94 ± 0.39	1.73 ± 0.12
SPC-Etho	1.75 ± 0.07	1.72 ± 0.01	1.57 ± 0.410	1.23 ± 0.31
SPC/chol-Etho	1.76 ± 0.15	1.64 ± 0.02	1.37 ± 0.11	1.23 ± 0.30

No permeation rate was observed with *t*-res alone during 24 h of incubation (data shown only until 6 h). When using transfersomes, *t*-res permeation through the skin was not observed, independently by the formulation used. On the contrary, in the case of ethanol-containing vesicles, an enhanced permeation rate was observed, dependently on the formulation. Actually, when the SPC-Etho was placed in contact with the skin, *t*-res permeation was observed after 1 h and 100% of the applied dosage accumulated in the receiving cell after 6 h. However, when using chol into the ethanol-containing vesicles formulation, any permeation rate was observed until 24 h (data shown only until 6 h). In Fig. 8, the amount of *t*-res found in the dermis following 24 h of skin incubation with the different samples is shown. A significant amount *t*-res, lower that 30 ng/mg of dermis, was found when incubating skin with a *t*-res solution. The use of transfersomes led to a decreased amount of *t*-res accumulated into the skin, that was about 12, 16 and 12 ng/mg of dermis, in case of Tw80-Trans, SDC-Trans and SC-Trans, respectively. A strong improvement of the *t*-res accumulated into the dermis, was found in the case of ethanol-containing vesicles, but only when chol was used in the nanocarriers, with about 45 ng/mg of dermis found after 24 h. SPC-Etho showed a *t*-res accumulation in the dermis comparable to that observed in the case of transfersomes (about 13 ng/mg dermis).

4. Discussion

In this study, we explored the possibility to develop nanocarriers conceived for topical administration of *t*-res. Due their properties to promote accumulation and/or passage of different drugs into/trough the skin, transfersomes (Cevc et al., 2008;) and ethanol-containing vesicles (Ainbinder et al., 2010) were taken into account in this study. In particular, different formulations based on *t*-res encapsulating transfersomes and ethanol-containing vesicles were developed. The first part of the study, the effect of vesicle

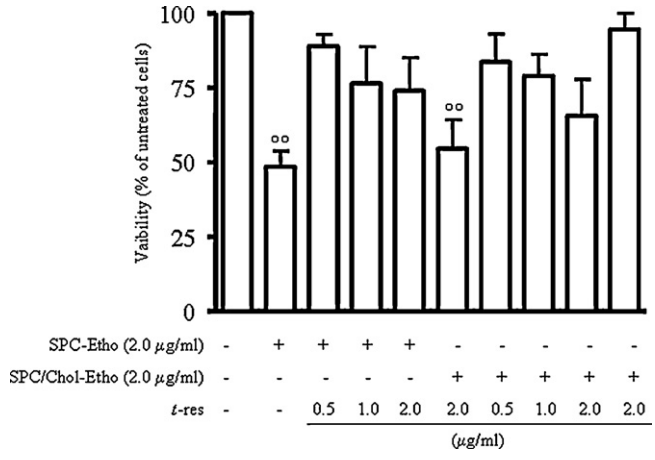

Fig. 2. MTT assay shows the effect of SPC or SPC/CHOL ethanol-containing vesicles, blank or encapsulating *t*-res, on cell viability. Data are mean ± S.E.M. of six experiments in triplicate.°° $p < 0.01$ *vs.* unstimulated cells.

composition on the technological characteristics of the formulations, with special attention to the vesicle stability during storage, was investigated. For comparison purpose, the two type of vesicles were similarly prepared by hydration of a lipid film with an aqueous solution (with or without ethanol), while method typically used to prepare ethosomes (Touitou et al., 2000) was avoid to limit difference in vesicle morphology. For this reason, in the case of vesicles prepared in presence of ethanol, we avoid the term of ethosomes and we preferred to use a more general term of ethanol-containing vesicles. Size characteristics of the vesicles depended on the type of nanocarriers and their composition. In the case of transfersomes, differences in mean diameter between the formulations were observed. Actually, transfersomes containing Tw80 showed a mean diameter significantly lower than SDC-Trans or SC-Trans. Moreover, SPC/Tw80 transfersomes were more heterogeneous in size, as indicated by the higher P.I. It has been already reported that the type of surfactant, actually the HLB, affect vesicle size (El Zaafarany et al., 2010). Moreover, it is worthy of note that in the preparation of SDC-Trans or SC-Trans, vesicle sizing was carried out by extrusion; in the case of vesicles containing Tw80, vesicle extrusion resulted in strong foaming and sizing by sonication was preferred. Thus, the type of surfactant, together with the sizing method could justify differences in size characteristics of transfersomes. In the case of ethanol-containing vesicles, any significant difference in size characteristics was observed between the two formulations. All formulations were stable following storage until two months at 4 °C.

All formulations had high encapsulation efficiency, especially in the case of transfersomes. The encapsulation process of a lipophilic drug into lipid vesicles should be driven by drug solubility into the lipid phase and partition coefficient between lipid phase and aqueous solution. The high solubility of *t*-res into the organic lipid solution, together with the low water solubility should contribute to the high actual loading into the transfersomes. However, the presence of 40% ethanol and the consequent higher *t*-res solubility into the hydration phase, could explain the lower *t*-res encapsulation found in the case of ethanol-containing vesicles. *t*-res solubility in the lipid phase should also favor its slow diffusion in the external phase during storage. This is the reason for that nanocarriers encapsulating *t*-res were freshly prepared before *in vitro* and *ex vivo* experiments.

The cytotoxicity of surfactants is well known, although a number of them have been approved for use as pharmaceutical excipients. In our experiments, we found toxicity in the case of all the formulations containing surfactants. This is expected due to the detergent activity of these molecules on the cell membrane. However, nanocarriers containing surfactant generally show lower toxicity than individual components (Müller et al., 1997). In our study, the nanocarriers showed a different cytotoxicity, depending on the type of surfactant and on the presence of *t*-res. Actually, a higher toxicity of blank Tw80-Trans, compared with blank SDC-Trans and blank SC-Trans, was found. A concentration-dependent toxicity of nanocarriers containing Tw80 on HaCaT cells has been already reported (Weyenberg et al., 2007). Taken together, these finding can be explained with the strong Tw80 detergent activity

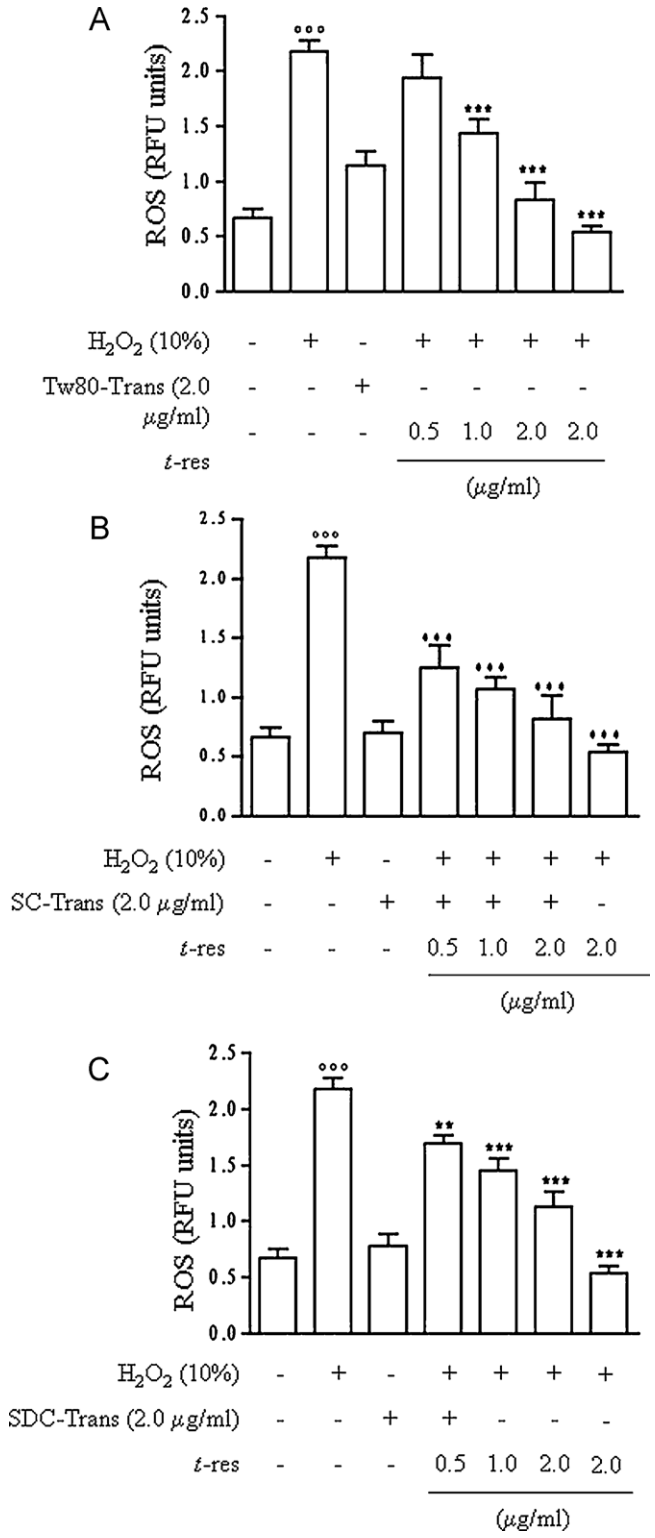

Fig. 3. Effect of Tw80 (A), SDC (B) or SC (C) transferosomes, blank or encapsulating *t*-res, on ROS generation in HaCat cells stimulated with H_2O_2 for 24 h. Data are mean ± S.E.M. of six experiments in triplicate.$^{\circ\circ\circ}p < 0.01$ *vs.* unstimulated cells; $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* H_2O_2.

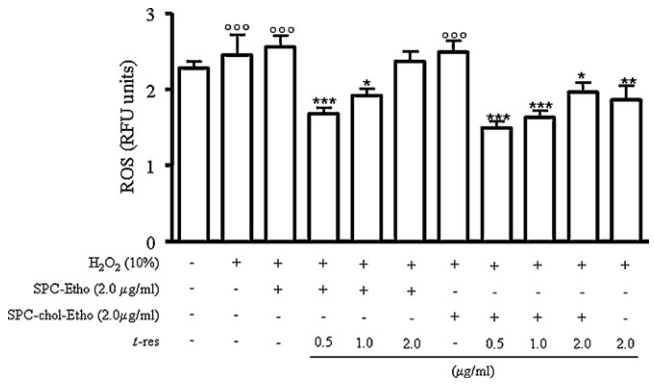

Fig. 4. Effect of SPC or SPC/chol ethanol-containing vesicles, blank or encapsulating *t*-res, on ROS generation in HaCat cells stimulated with H_2O_2 for 24 h. Data are mean ± S.E.M. of six experiments in triplicate.$^{\circ\circ\circ}p < 0.01$ *vs.* unstimulated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* H_2O_2.

on cell membrane, that can undergo autoxidation with formation of high ROS concentrations. This is in agreement with already reported data (Yao et al., 2009). Indeed, ROS production induced by blank Tw80-Trans were found in our experiments in absence of oxidant stimulus (i.e. H_2O_2 challenge). This effect was not observed in the case of blank SDC-Trans and blank SC-Trans. This hypothesis was also confirmed by MDA assay in which we found high lipid peroxidation when cells were incubated with blank Tw80-Trans. Once more, this effect was not found when cells were incubated with blank SDC-Trans and blank SC-Trans. However, when using Tw80-Trans, we observed a strong reduction of ROS production as well as reduction of lipid peroxidation, compared with blank nanocarriers. This is in agreement with the reduced toxicity observed with Tw80-Trans, compared with blank Tw80-Trans. In the case ethanol-containing vesicles, a significant cytotoxicity was observed with blank SPC-Etho and blank SPC/chol-Etho. Moreover, when cells were incubated with *t*-res-containing ethanol-containing vesicles, namely SPC-Etho and SPC/chol-Etho, a lower toxicity was observed in concentration depending manner. These results reflect findings on ROS production, in which high ROS concentration was found following cell incubation with blank ethanol-containing vesicles. Again, ROS production was lower when incubating cells with SPC-Etho and SPC/chol-Etho, especially at high *t*-res concentration. Thus, ethanol-containing vesicles cytotoxicity can certainly be ascribed to the induction of ROS production, that can be limited by presence of *t*-res. It is worthy of note that, differently than Tw80 containing carriers, the cytotoxicity of ethanol-containing vesicles as well as the induction of ROS production did not relate to the lipid peroxidation, that was reduced by presence of ethanol-containing vesicles, blank or containing *t*-res. It is reasonable to hypothesize that, in the case of ethanol-containing vesicles, the ethanol is the main responsible of the cytotoxicity. Our data underline that a different mechanism should be ascribed to justify the different toxicity observed in the case of Tw80 and ethanol containing nanocarriers. Further experiments should clarify this aspect.

Transfersomes and ethanol-containing vesicles have been shown to enhance the percutaneous delivery and accumulation of drugs into the skin, as compared with liposomes (Rao et al., 2004; Fang et al., 2008). The edge activators or ethanol may enhance the deformability of the lipid bilayer. Alternatively, the interaction between ethanol or edge activator and lipids in the *stratum corneum* may decrease the transformation temperature of the latter, increase its fluidity, and promote drug permeation (Rao et al., 2004; Fang et al., 2008). In our study, *ex vivo* measurements demonstrated that only the SPC-Etho facilitated the transfer of resveratrol across samples of porcine skin just after 6 h, whereas *t*-res permeation was not observed when it was simply dissolved in water and placed in contact with the skin samples. The use of transfersomes reduced *t*-res accumulation into the dermis, with an effect dependent on the type of surfactant used in the formulation. In the case of ethanol-containing vesicles, *t*-res accumulation was lowered or enhanced in the case of SPC-Etho and SPC/chol-Etho, respectively.

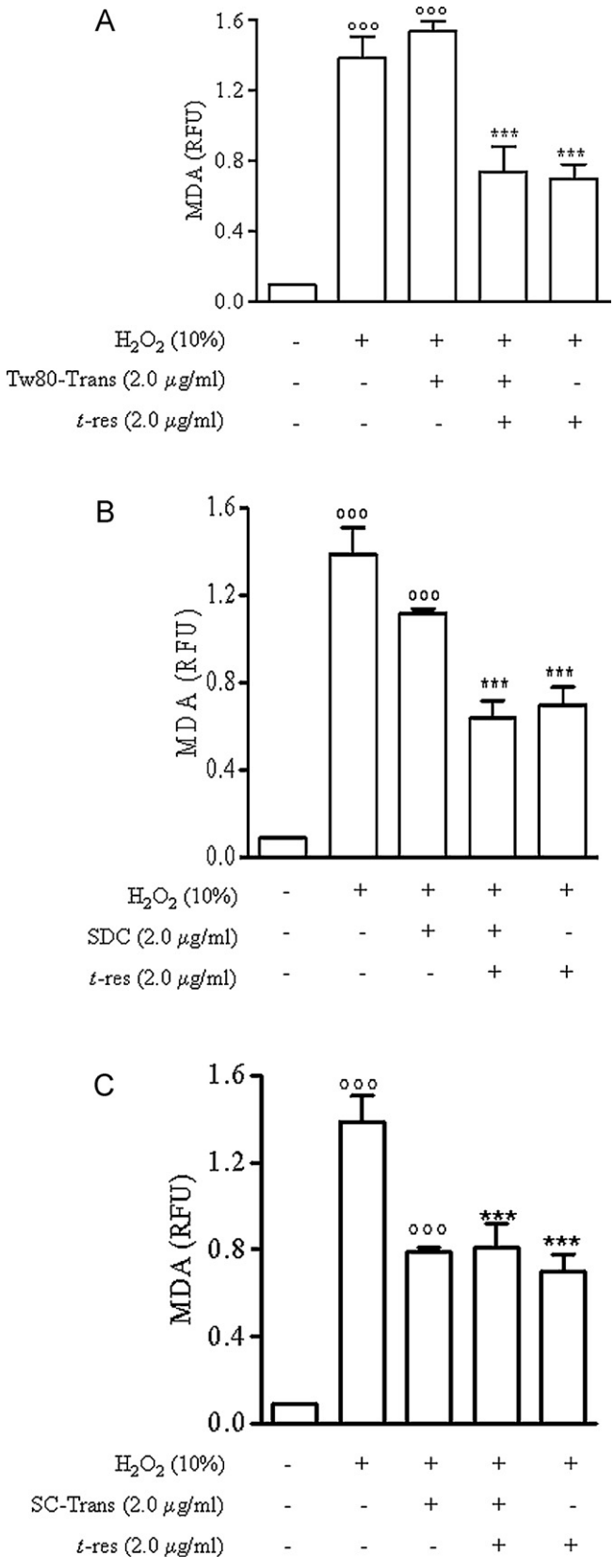

Fig. 5. Effect of Tween80 (A), SDC (B) or SC (C) transferosomes, blank or encapsulating *t*-res, on lipid peroxidation in HaCat cells stimulated with H_2O_2 for 24 h. Data are mean ± S.E.M. of six experiments.°°°$p < 0.01$ *vs.* unstimulated cells; ***$p < 0.001$ *vs.* H_2O_2.

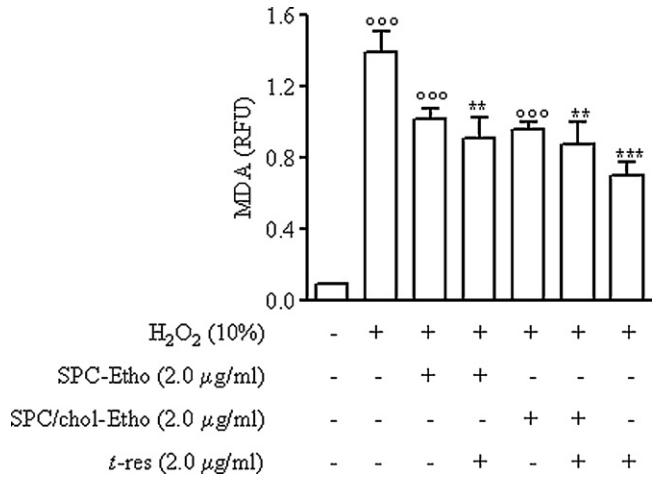

Fig. 6. Effect of SPC or SPC/chol ethanol-containing vesicles, blank or encapsulating *t*-res, on lipid peroxidation in HaCat cells stimulated with H_2O_2 for 24 h. Data are mean ± S.E.M. of six experiments.°°°$p < 0.01$ *vs.* unstimulated cells; **$p < 0.01$, ***$p < 0.001$ *vs.* H_2O_2.

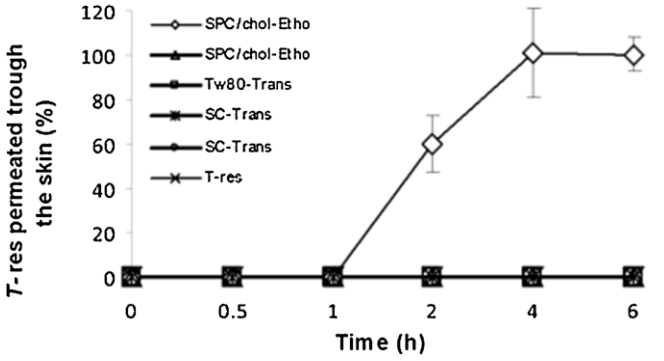

Fig. 7. *t*-res permeation through excised skin from pig ear, following incubation with *t*-res in solution or encapsulated into the nanocarriers.

From this data it is possible to speculate that ethanol and fluidity of vesicle bilayer could be both essential to promote *t*-res permeation though the skin. In fact, the use of ultraflexible liposomes, such as transfersomes, was not sufficient to achieve *t*-res passage, leading to reduced *t*-res accumulation in dermis and no permeation through the skin. in the case of ethanol-containing vesicles, an increased *t*-res accumulation into the dermis was observed. In particular, in the case of SPC/chol-Etho, *t*-res accumulation into the

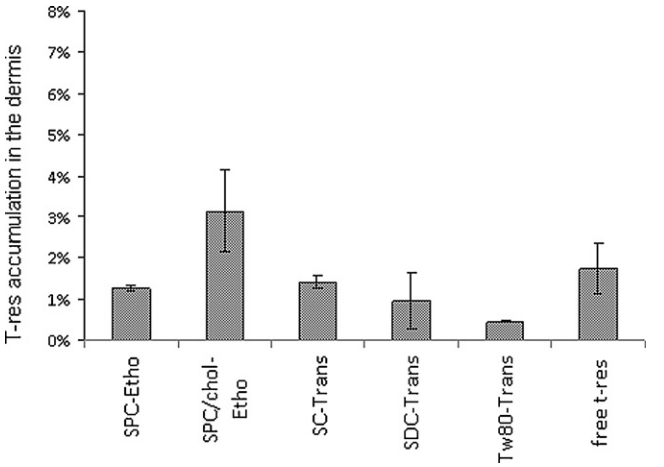

Fig. 8. *t*-res accumulation in the dermis after 24 of excised skin incubation with *t*-res in solution or encapsulated into the nanocarriers.

dermis was enhanced, when compared with skin incubated with a *t*-res in solution. The higher fluidity of SPC-Etho due to the absence of chol in the bilayer, should facilitate the enhancer effect of ethanol into the vesicles, thus allowing not only *t*-res accumulation into the dermis, but also promoting *t*-res permeation through the skin.

5. Conclusions

In this study, the potential of transfersomes and ethanol-containing vesicles to deliver resveratrol through the skin has been investigated. In particular, we demonstrated that with both the type of nanocarrier a high *t*-res encapsulation efficiency can be achieved. The toxicity of the nanocarrier can be strongly affected by the surfactant used in the formulation. Polysorbate 80 as well as ethanol were certainly the most dangerous for cell viability *in vitro*. However, the presence of *t*-res can have a protective effect, reducing ROS formation as well as formation of lipid peroxides. Finally, only ethanol-containing vesicles based on SPC were able to promote *t*-res passage through the skin in *ex vivo* experiments. This study should contribute to understand the effect of the formulation on skin permeation of *t*-res, a molecule for a which there is a growing interest, especially for its dermatological applications. Moreover, from our finding, a new possible application for ethanol-containing vesicles can be proposed. Of course, stability issues need to be addressed before to propose this kind of formulation for commercial applications.

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References

Ainbinder, Touitou, 2005. Testosterone ethosomes for enhanced transdermal delivery. Drug Deliv. 12 (September–October (5)), 297–303.
Ainbinder, D., Paolino, D., Fresta, M., Touitou, E., 2010. Drug delivery applications with ethosomes. J. Biomed. Nanotechnol. 6 (October (5)), 558–568.
Baur, J.A., Sinclair, D.A., 2006. Therapeutic potential of resveratrol: the *in vivo* evidence. Nat. Rev. Drug Discov. 5 (June (6)), 493–506.
Bhat, K.P., Pezzuto, J.M., 2002. Cancer chemopreventive activity of resveratrol. Ann. N. Y. Acad. Sci. 957 (May), 210–229.
Cevc, G., Blume, 1992. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim. Biophys. Acta 1104 (February (1)), 226–232.
Cevc, G., Blume, G., 2001. New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, transfersomes. Biochim. Biophys. Acta 1514 (October (2)), 191–205.
Cevc, G., Gebauer, D., 2003. Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. Biophys. J. 84 (February (2 Pt 1)), 1010–1024.
Cevc, G., Mazgareanu, S., Rother, M., 2008. Preclinical characterisation of NSAIDs in ultradeformable carriers or conventional topical gels. Int. J. Pharm. 360 (August (1–2)), 29–39.
Cevc, G., 1996. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 13 (3–4), 257–388.
Chan, M.M., 2002. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochem. Pharmacol. 63 (January (2)), 99–104.
Chen, X., He, H., Wang, G., Yang, B., Ren, W., Ma, L., Yu, Q., 2007. Stereospecific determination of *cis*- and *trans*-resveratrol in rat plasma by HPLC: application to pharmacokinetic studies. Biomed. Chromatogr. 21 (March (3)), 257–265.
Dayan, N., Touitou, E., 2000. Carriers for skin delivery of trihexyphenidyl HCl: ethosomes *vs.* liposomes. Biomaterials 21 (September (18)), 1879–1885.
De Stefano, D., Maiuri, M.C., Simeon, V., Grassia, G., Soscia, A., Cinelli, M.P., Carnuccio, R., 2007. Lycopene, quercetin and tyrosol prevent macrophage activation induced by gliadin and IFN-gamma. Eur. J. Pharmacol. 566 (July (1–3)), 192–199.
Detoni, C.B., Souto, G.D., da Silva, A.L., Pohlmann, A.R., Guterres, S.S., 2012. Photostability and skin penetration of different e-resveratrol-loaded supramolecular structures. Photochem. Photobiol. 88 (July (4)), 913–921.
Docherty, J.J., Fu, M.M., Hah, J.M., Sweet, T.J., Faith, S.A., Booth, T., 2005. Effect of resveratrol on herpes simplex virus vaginal infection in the mouse. Antiviral Res. 67 (September (3)), 155–162.
Dragicevic-Curic, N., Gräfe, S., Gitter, B., Winter, S., Fahr, A., 2010. Surface charged temoporfin-loaded flexible vesicles: *in vitro* skin penetration studies and stability. Int. J. Pharm. 384 (January (1–2)), 100–108.
El Maghraby, G.M., Williams, A.C., Barry, B.W., 2000a. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. Int. J. Pharm. 196 (February (1)), 63–74.
El Maghraby, G.M., Williams, A.C., Barry, B.W., 2000b. Skin delivery of oestradiol from lipid vesicles: importance of liposome structure. Int. J. Pharm. 204 (August (1–2)), 159–169.
El Zaafarany, G.M., Awad, G.A., Holayel, S.M., Mortada, N.D., 2010. Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. Int. J. Pharm. 397 (September (1–2)), 164–172.
Elsayed, M.M., Abdallah, O.Y., Naggar, V.F., Khalafallah, N.M., 2007a. Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. Pharmazie 62 (February (2)), 133–137.
Elsayed, M.M., Abdallah, O.Y., Naggar, V.F., Khalafallah, N.M., 2007b. PG-liposomes: novel lipid vesicles for skin delivery of drugs. J. Pharm. Pharmacol. 59 (October (10)), 1447–1450.
Fabbrocini, G., Staibano, S., De Rosa, G., Battimiello, V., Fardella, N., Ilardi, G., La Rotonda, M.I., Longobardi, A., Mazzella, M., Siano, M., Pastore, F., De Vita, V., Vecchione, M.L., Ayala, F., 2011. Resveratrol-containing gel for the treatment of acne vulgaris: a single-blind, vehicle-controlled, pilot study. Am. J. Clin. Dermatol. 12 (April (2)), 133–141, http://dx.doi.org/10.2165/11530630-000000000-00000.
Fan, E., Zhang, K., Jiang, S., Yan, C., Bai, Y., 2008. Analysis of *trans*-resveratrol in Grapes by micro-high performance liquid chromatography. Anal. Sci. 24 (8), 1019–1023.
Fang, Y.P., Tsai, Y.H., Wu, P.C., Huang, Y.B., 2008. Comparison of 5-aminolevulinic acid-encapsulated liposome versus ethosome for skin delivery for photodynamic therapy. Int. J. Pharm. 356 (May (22)), 144–152.
Honeywell-Nguyen, P.L., Gooris, G.S., Bouwstra, J.A., 2004. Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin *in vivo*. J. Invest. Dermatol. 123 (November (5)), 902–910.
Hrenn, A., Steinbrecher, T., Labahn, A., Schwager, J., Schempp, C.M., Merfort, I., 2006. Plant phenolics inhibit neutrophil elastase. Planta Med. 72 (October (12)), 1127–1131.
Hung, C.F., Lin, Y.K., Huang, Z.R., Fang, J.Y., 2008. Delivery of resveratrol, a red wine polyphenol, from solutions and hydrogels via the skin. Biol. Pharm. Bull. 31 (May (5)), 955–962.
Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., Pezzuto, J.M., 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275 (January (5297)), 218–220.
Khanna, S., Venojarvi, M., Roy, S., Sharma, N., Trikha, P., Bagchi, D., Bagchi, M., Sen, C.K., 2002. Dermal wound healing properties of redox-active grape seed proanthocyanidins. Free Radic. Biol. Med. 33 (October (8)), 1089–1096.
López-Pinto, J.M., González-Rodríguez, M.L., Rabasco, A.M., López-Pinto, J.M., González-Rodríguez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int. J. Pharm. 298 (July (1)), 1–12.
Müller, R.H., Rühl, D., Runge, S., Schulze-Forster, K., Mehnert, W., 1997. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. Pharm. Res. 14 (April (4)), 458–462.
Orallo, F., 2006. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. Curr. Med. Chem. 13 (1), 87–98.
Paolino, D., Lucania, G., Mardente, D., Alhaique, F., Fresta, M., 2005. Ethosomes for skin delivery of ammonium glycyrrhizinate: *in vitro* percutaneous permeation through human skin and *in vivo* anti-inflammatory activity on human volunteers. J. Control. Release 106 (August (1–2)), 99–110.
Rao, G.C., Kumar, M.S., Mathivanan, N., Rao, M.E., 2004. Nanosuspensions as the most promising approach in nanoparticulate drug delivery systems. Pharmazie 59 (January (1)), 5–9.
Stewart, J.C., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104 (May (1)), 10–14.
Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M., 2000. Ethosomes – novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Release 65 (April (3)), 403–418.
Trotta, M., Peira, E., Carlotti, M.E., Gallarate, M., 2004. Deformable liposomes for dermal administration of methotrexate. Int. J. Pharm. 270 (February (1–2)), 119–125.
van den Bergh, B.A., Wertz, P.W., Junginger, H.E., Bouwstra, J.A., 2001. Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. Int. J. Pharm. 217 (April (1–2)), 13–24.
Weyenberg, W., Filev, P., Van den Plas, D., Vandervoort, J., De Smet, K., Sollie, P., Ludwig, A., 2007. Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application. Int. J. Pharm. 337 (June (1–2)), 291–298.
Yao, J., Dokuru, D.K., Noestheden, M., Park, S.S., Kerwin, B.A., Jona, J., Ostovic, D., Reid, D.L., 2009. A quantitative kinetic study of polysorbate autoxidation: the role of unsaturated fatty acid ester substituents. Pharm. Res. 26 (October (10)), 2303–2313.