International Journal of Pharmaceutics 363 (2008) 183–191

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells

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ARTICLE INFO

Article history:
Received 21 April 2008
Received in revised form 14 July 2008
Accepted 19 July 2008
Available online 31 July 2008

Keywords:
Resveratrol
Liposomes
UV-B irradiation
Cell proliferation
Photoprotection
Toxicity

ABSTRACT

The possibility of improving the efficacy of resveratrol, a polyphenol with strong antioxidant and free-radical scavenging properties, on cell proliferation and photoprotection by liposomal incorporation was investigated. Oligolamellar vesicles of different lipid compositions, loaded with resveratrol, were prepared and characterized by evaluating size, zeta potential, incorporation efficiency, electron microscopy and stability over 60 days. The effect of free and liposomal resveratrol on the viability of HEK 293 cells and their photoprotection after UV-B irradiation was assessed by the MTS method. Resveratrol decreased the cell viability at 100 μM concentration, while at 10 μM increased cell proliferation and also achieved the most effective photoprotection. Photomicrographs of the treated cells from inverted light and fluorescence microscopy demonstrated resveratrol effectiveness at 10 μM, as well as its toxicity at higher concentrations, based on changes in cell shape, detachment and apoptotic features. Interestingly, liposomes prevented the cytotoxicity of resveratrol at high concentrations, even at 100 μM, avoiding its immediate and massive intracellular distribution, and increased the ability of resveratrol to stimulate the proliferation of the cells and their ability to survive under stress conditions caused by UV-B light.

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, Fig. 1) is a naturally occurring polyphenolic phytoalexin synthesized by a wide variety of plant species, including grapes (*Vitis* spp.), berries (*Vaccinium* spp.) and peanuts (*Arachis* spp.), in response to stress as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation (UV) (Langcake and Pryce, 1976; Signorelli and Ghidoni, 2005).

Resveratrol has been shown to possess an exceptionally potent antioxidant activity, even stronger than vitamin E and C in some assay systems (Cadenas and Barja, 1999; Stojanovic et al., 2001), and furthermore, an extremely interesting dual action. It has been reported to participate in prosurvival as well as prodeath cellular mechanisms, depending on cellular conditions, specific cell molecular settings and the concentration used (Signorelli and Ghidoni, 2005). Resveratrol inhibits the oxidation of low-density lipoproteins and platelet aggregation (Frankel et al., 1993; Pace-Asciak et al., 1995; Wang et al., 2002); it has a strong anti-inflammatory property acting through the inhibition of cyclo-oxygenases (Pace-Asciak et al., 1995; Jang et al., 1997) and it has been demonstrated to exert striking cancer chemopreventive activity. Jang et al. (1997) have shown that topical application of resveratrol inhibited chemically-induced skin tumorigenesis in CD-1 mice, with no manifest signs of drug-induced toxicity. Since then, several studies have been carried out on different human cancer cell lines and in animal carcinogenesis models, confirming the striking chemopreventive and chemotherapeutic abilities of resveratrol (Mgbonyebi et al., 1998; Gusman et al., 2001; Schneider et al., 2001; Joe et al., 2002; Aggarwal et al., 2004).

In regard to skin cancer, the chronic exposure of the skin to UV-B radiation (280–320 nm) is reported to be a tumour initiator, promoter and co-carcinogen, as it results in a variety of biological responses directly or indirectly related to the excessive production of reactive oxygen species (ROS) (Urbach, 1978; Ichihashi et al., 2003; Katiyar, 2007). ROS are normal byproducts of cellular physiology, continuously removed by enzymatic and non-enzymatic antioxidants that scavenge the radicals, preventing them from attacking the biological targets and thus maintaining a prooxidant/antioxidant balance. However, extensive and chronic exposure of the skin to UV radiation generates high levels of ROS which overwhelm skin cells, react with DNA, proteins and fatty acids, with consequent alteration of cell structure, metabolism, differentiation and proliferation that may result in various

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doi:10.1016/j.ijpharm.2008.07.024

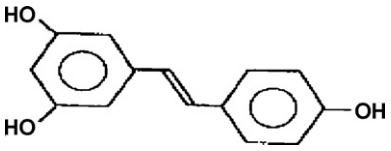

Fig. 1. Chemical structure of resveratrol.

pathological conditions, such as immunosuppression, inflammation, skin aging and cancer (Black et al., 1997; Kohen, 1999; F'guyer et al., 2003; and references therein).

In recent years several studies have focused on novel approaches to protect human skin from UV radiation and to reduce the occurrence of cutaneous malignancies by the use of dietary antioxidants, among which resveratrol is one of the most promising (Afaq et al., 2003; F'guyer et al., 2003; Howitz et al., 2003). Indeed, recent papers highlight the ability of resveratrol to suppress, retard or reverse the deleterious effects of UV radiation, thus favouring preservation of the functional status of cells and extending cell lifespan (Signorelli and Ghidoni, 2005). Several authors showed that pretreatment of different cell lines with resveratrol promotes cell survival and protects from radiation-induced apoptosis, as well as it affords protection against the UV-B damage caused to the skin of animal models, in a dose- and time-dependent manner, by its strong antioxidant properties (Adhami et al., 2003; Afaq et al., 2003; Howitz et al., 2003).

In most experiments resveratrol has been used in a free form dissolved in different organic solvents (i.e., DMSO or acetone) that are not adequate for delivery. Since it is a poorly water soluble drug (<0.001 mol/l), weakly absorbed after oral administration, and unstable as it converts to the *cis*-form (a less active form) particularly on exposure to UV light (Fremont, 2000; Aggarwal et al., 2004), topical delivery using liposomes is a good option in order to overcome all these limitations. Indeed, liposomes are optimal carrier for the entrapment and cellular delivery of drugs because they can incorporate a lipophilic drug within the membrane bilayers, thus protecting it from light and other degradative processes (Düzgüne and Nir, 1999; Kristl et al., 2003; Šentjurc et al., 2004; Padamwar and Pokharkar, 2006). They also enable slow release at the target site over a prolonged period of time (Manconi et al., 2007). Therefore, in the present study liposomes were designed for effective delivery of resveratrol to cells and their effect on the survival and proliferation of the cells under normal and stress conditions caused by UV-B exposure was assessed and compared to that of free resveratrol. Different resveratrol liposomal formulations were developed, characterized and tested on HEK 293 cells. Furthermore, the morphological changes of the cells treated by free or loaded resveratrol and the localization of liposomes in the cells were investigated.

2. Materials and methods

2.1. Materials

Resveratrol (RSV, >99% pure), cholesterol, dicetyl phosphate (DCP), and lecithin (type IV-S; soybean phosphatidylcholine ≥30% pure) were from Sigma (Germany); ATX Tris buffer was purchased from Fluka (Germany); enriched soy phosphatidylcholine (Phospholipon90G, P90G, phosphatidylcholine 92–98% pure) was supplied by Natterman Phospholipids (Germany).

2.2. Methods

2.2.1. Liposome preparation

Different liposomal formulations were prepared, made from P90G, DCP, cholesterol or lecithin alone, empty or loaded with resveratrol (see Table 1). All components were weighted in a glass flask, one after the other, 5 ml of Tris–HCl were added, the pH adjusted to 7.4 and then the suspension was sonicated with a high intensity ultrasonic processor equipped with a tapered microtip (Cole-Parmer, USA), until a clear opalescent suspension was obtained. To produce small oligolamellar vesicles, the liposome suspensions were forced 21 times through 19-mm polycarbonate filters (100 nm pore size) in a LiposoFast™ extruder (Avestin, Canada). Liposomal suspensions were then loaded into dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories, Inc., USA) and dialysed against distilled water. Dialysis of each sample (5 ml) was carried out in 1000 ml of water for 2 h, which were appropriate to allow the dissolution and consequent removal of the non-entrapped resveratrol, and to avoid the destabilization of the vesicular suspension (i.e., osmotic swelling and vesicle fusion) as well. All suspensions were prepared and kept in the dark all the time.

Table 1
Composition of resveratrol-loaded liposomes

Component (μmoles per 5 ml of suspension)	Sample	
	A	B
P90G	129.0	
Lecithin		129.0
DCP	36.0	
Cholesterol	26.0	
RSV	6.5	6.5

P90G, phospholipon 90G; DCP, dicetyl phosphate; RSV, resveratrol.

To visualize the liposomes within the cells by fluorescence microscopy, green-fluorescent coumarin-6 (Sigma, Germany) was added to the lipophilic components before sonication.

2.2.2. Liposome characterization

The average size, polydispersity index (P.I.) and zeta potential of the liposomal vesicles were measured by dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The nano-ZS systematically and automatically adapts to the sample by adjusting the intensity of the laser and the attenuator of the photomultiplier, thus ensuring reproducibility of the experimental measurement conditions.

Vesicle formation and morphology were checked by transmission electron microscopy (TEM) using a Philips CM 100 microscope (Amsterdam, the Netherlands) operating at 80 kV. Liposomes were examined using a negative staining technique: samples were adsorbed on a carbon grid and stained with 2.5% (w/v) ammonium molybdate. Pictures were recorded by BioScan CCD Camera using Digital Micrograph software (Gatan Inc., Washington, DC, USA).

The incorporation efficiency (*E*%) of the different purified vesicular formulations was expressed as the percentage of the amount of resveratrol initially used. Free resveratrol was removed from liposome dispersions by dialysis (as described in Section 2.2.1). After this purification the liposomes were disrupted with 0.025% non-ionic Triton X-100 and the resveratrol content was quantified by HPLC. Quantitative analyses were carried out at 306 nm using an Agilent 1100 Series HPLC System (Germany) equipped with a diode array detector. The analytical column was an XBridge C18, 5 μm, 4.6 mm × 150 mm (Waters, USA). The mobile phase was a mixture of methanol, acetonitrile, water and acetic acid (75:22.5:2.4:0.1, v/v) at a flow rate of 0.8 ml/min.

Liposome stability was evaluated by monitoring size and surface charge over 60 days at 4 °C. Resveratrol retention and chemical stability during storage (in hydrating buffer) were also checked by

measuring the amount of loaded resveratrol, after dialysis, by HPLC over 60 days.

2.2.3. Preparation of antioxidant samples

10 mM stock ethanolic solutions of resveratrol were prepared and stored at −20 °C. Working concentrations were prepared by diluting the stock in culture medium. Likewise, resveratrol-loaded liposomes were diluted in culture medium as required. Empty liposomes, added to the cell cultures at the same volume as resveratrol-loaded liposomes, served as a control.

2.2.4. Cell culture and treatment with antioxidants

Human embryonic kidney cells (HEK 293; American Type Culture Collection ATCC, Manassas, VA, USA), were used as model cells since they are epithelial adherent, like normal human keratinocytes, but much easier to culture and to manipulate. They were grown as adherent cultures in 75 cm^2 culture flasks using, as growth medium, Dulbecco's-modified Eagle medium (DMEM; Sigma, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco®, Invitrogen, USA), 1.0% 200 mM L-glutamine and 1.0% antibiotic/antimycotic (Sigma, Germany) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

HEK 293 cell suspensions were prepared as follows: culture medium was removed from the flasks and the cells were rinsed three times with fresh medium and resuspended. Cells were counted with a hemocytometer, diluted to a density of 2×10^5 cells/ml and seeded by transferring the cell dilutions into 96-well plates at 100 μl-well. After 24 h of attachment, medium was replaced and resveratrol or liposomal formulations were added to the wells: resveratrol (RSV) at 10, 50 and 100 μM, empty liposomes, and resveratrol-loaded liposomes (RSV 10 and 100 μM). Control cultures received the same volume of supplemented DMEM and were used as a cell-only control (CC). Further, dilutions of resveratrol and liposomes (as above) were seeded into the culture plates at 100 μl-well without cells as background control samples (BC). In addition, a sample of culture medium was used as a medium-only control (MC). The effect of ethanol on the cells, investigated by incubating the cultures with 10 μl of ethanol per well, was detected. For each sample and control, three replicates were tested. After 24 h of incubation, cell viability was assessed by MTS assay.

2.2.5. MTS assay

The MTS assay is an economical, rapid, sensitive and specific colorimetric method for assessing cell viability and *in vitro* cytotoxicity. This method, which offers several advantages over other cytotoxicity tests like MTT (Malich et al., 1997; Si et al., 1999), is based on the reduction, by mitochondrial dehydrogenase in metabolically active cells, of the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium inner salt (MTS), to the coloured, water-soluble formazan that absorbs at 490 nm. Since the amount of formazan is directly proportional to the number of living cells in a culture, the intensity of the produced colour is an indication of the viability of the cells: reduction of this intensity thus indicates cellular damage.

For the MTS assay, 20 μl of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA), which is composed of the novel tetrazolium compound MTS and an electron coupling reagent phenazine ethosulfate (PES, a redox intermediary), was added to each well according to the manufacturer's instructions. After 3 h in culture, the cell viability was determined by measuring the absorbance at 490 nm using a Safire^{2TM} microplate reader (TECAN, Switzerland). Results are expressed as a percentage of the viability in control cells incubated in medium alone according to the following formula:

$$\text{Viability}\,(\%) = \left(\frac{A_{CS} - A_{BC}}{A_{CC} - A_{MC}}\right) \times 100$$

where A is the absorbance at 490 nm (mean of three replicates): A_{CS}, is the cells treated with the sample; A_{BC}, is the background control sample (sample in medium); A_{CC}, is the cell-only control; A_{MC}, is the medium-only control.

2.2.6. UV irradiation of cells

To assess the effect of resveratrol on survival of HEK 293 cells after UV-B irradiation a UV-B source ENF-260C/FE Spectroline® UV lamp (Spectronics Corporation, Westbury, NY, USA) was used. Cells were seeded at 2×10^5 cells/ml, allowed to attach for 24 h and then treated with the investigated samples for 24 h, as described in Section 2.2.4. After that, the cells were exposed to UV-B light (350 μW/cm^2) for 1, 2 or 3 h (each hour corresponding to 1.26 J) and MTS assay was performed. Control cells were irradiated, but not treated.

2.2.7. Morphological examination of cells

The morphology of HEK 293 cells was examined by inverted light and fluorescence microscopy. Cells (1×10^5 cells/ml) were plated on square glass cover slips in 6-well plates in supplemented DMEM medium (as described in Section 2.2.4) in the absence (control) or presence of the investigated samples. After 24 h of incubation, cells were exposed to UV-B radiation for 1 h or not irradiated (negative control) and examined under the inverted microscope (Olympus CKX41, Tokyo, Japan).

For fluorescence microscopy, medium was removed from each well, the cells were fixed with ice-cold 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.1% Triton X-100. The fixed cells were washed in PBS and stained with the blue-fluorescent DNA stain Hoechst 33258 (Sigma, Germany) to visualize the cell nucleus and the green-fluorescent dye coumarin-6 or the red-fluorescent dye phalloidin rhodamine (Sigma, Germany) (for the cells treated with green-fluorescent liposomes) for staining the actin cytoskeleton in HEK 293 cells. After staining, the adhering cells were washed in PBS, air dried and covered with ProLong antifade (Molecular Probes^{TM}, Invitrogen, USA). Cover slips were then mounted on the slide which was observed under the fluorescence microscope (Olympus IX81, Tokio, Japan). Picture were taken in z-stacks using the 60-fold objective with the disc scanning unit (DSU) to avoid out of focus stray light, and processed by the CellR̂ Software (Olympus, Tokio, Japan).

2.2.8. Statistical analysis

All data are expressed as the mean ± S.D. of at least three independent experiments. Differences between samples were examined statistically using the appropriate non-parametric test (the unpaired, two-tailed Student's *t*-test). Significance was tested at the 0.05* and 0.001** levels of probability.

3. Results and discussion

3.1. Preparation of liposomes for the delivery of resveratrol and their physicochemical characterization

Two different liposomal formulations were developed to achieve the optimal system for the entrapment and delivery of resveratrol. No substantial differences were observed between liposomes made from different lipid compositions (type of phospholipid). Liposomes were obtained by extrusion, which is reported to be the most suitable technique to get homogeneous distribution of vesicle size (Budai et al., 2004). Indeed, the extruded liposomes

Table 2
Characteristics of resveratrol formulations: liposome size, polydispersity index (P.I.), zeta potential and resveratrol incorporation efficiency (*E*%)

Sample	Size (nm)	P.I.	Zeta potential (mV)	*E* (%)
A	70.7 ± 1.9	0.237 ± 0.031	−38.4 ± 0.6	79.7 ± 3.8
B	99.5 ± 0.3	0.270 ± 0.020	−44.5 ± 0.4	73.1 ± 3.3

Each value represents the mean ± S.D., $n = 3$. The composition of samples is given in Table 1.

were uniform in size, with an average diameter close to the filter pore size of the extruder (100 nm) and P.I. was ∼0.2 (Table 2). The P.I. is a mathematical definition accounting for the relative error between curve fit and experimental values (Pusey and van Megen, 1989), which characterizes the homogeneity of the colloidal suspensions. P.I. values greater than 0.7 indicate that sample has a very broad size distribution. According to this, our results disclose a narrow size distribution, hence a good quality for all formulations. The zeta potential was negative (Table 2), most likely due to the presence of DCP in formulations A, and to the heterogeneous anionic lipidic fractions in lecithin (phosphatidylserine, phosphatidic acid, phosphatidylglycerol, phosphatidylinositol) in formulation B.

Resveratrol incorporation into liposomes at a percentage higher than 70 (Table 2) was achieved by all liposomal formulations, showing their good incorporation capability, which was not significantly influenced by the investigated lipid composition. TEM analysis showed small, spherical and oligolamellar vesicles (Fig. 2a and b). The two investigated phospholipids did not influence the vesicle morphology. The stability of the prepared formulations, evaluated by measuring the zeta potential and the average size, showed no significant changes during storage time. The zeta potential is a good index of the magnitude of the repulsive interaction between colloidal particles and is commonly used to assess the stability of a vesicular suspension. If the particles have and maintain a large negative or positive zeta potential, they will repel each other and the suspension will be stable. In particles with low zeta potential, there is only a little repulsion force and the particles will eventually aggregate, resulting in suspension instability. During this study, zeta potential measurements showed no significant changes on storage and size analyses revealed that there were no changes (i.e., increase in size due to aggregation) in the long-term stability study, thus providing evidence of the stability of our formulations.

Finally, the amount and the chemical stability of resveratrol in liposomes was investigated by HPLC over a 60-day period at 4 °C. The amount of resveratrol loaded did not change and no peak was detected at 288 nm (absorption λ of *cis*-resveratrol), indicative of *trans*/*cis*-isomerization.

3.2. Evaluation of the cell proliferative response to resveratrol under normal conditions

The effect of resveratrol on the growth (survival and proliferation) of HEK 293 cells, assessed by MTS method, is shown in Fig. 3a. It is noteworthy that the amount of ethanol used for the sample dilutions in our experiments did not interfere with the cell viability, as was evidenced by MTS assay (data not reported). Since a very broad range of resveratrol testing concentration is found in the literature (from 0.5 to 200 μM or more), we opted for 100 μM as a reasonably effective concentration and as a starting point for our experiments. Therefore, cells were treated with 100 μM resveratrol for 24 h. Unexpectedly, resveratrol in this concentration strongly inhibited cell metabolic activity (Fig. 3a). In the light of the finding, we incubated the cells for 24 h with two lower resveratrol concentrations (10 and 50 μM). The resveratrol promotion of cell activity was inversely proportional to the dose: the lowest tested concentration activated the cells, whereas already at 50 μM dose a slightly deteriorative effect was observed. Such opposing effects, depending on resveratrol concentration, may be explained as a function of its liposolubility that leads to an accumulation in the cell membrane where, at high concentration, it probably acts as a prooxidant, affecting cellular physiology and leading to cell death. This is the common postulated mechanism underlying resveratrol's anti-tumour effect. Therefore, resveratrol can behave both as antioxidant and prooxidant, depending on its concentration and also on cell type and cytosolic redox status, as reported by other groups (Szende et al., 2000; Michels et al., 2006, and references therein; Alarcón de la Lastra and Villegas, 2007).

Direct microscopic observation of the cells under the inverted light microscope clearly documented the effect of resveratrol on the cells, and supported the MTS results (Fig. 3). Cells treated with resveratrol at 100 μM were reduced in number, compared with

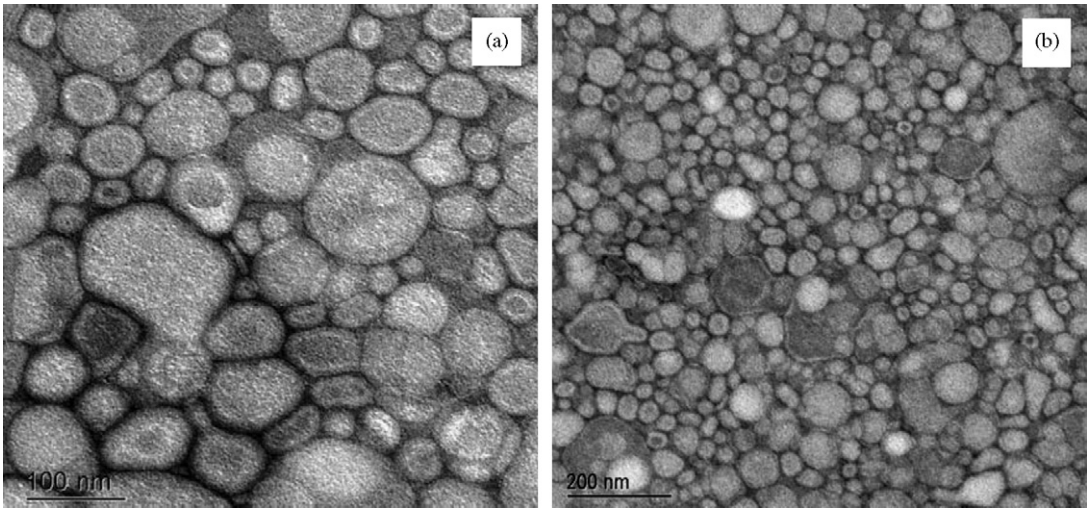

Fig. 2. (a and b) TEM micrographs of resveratrol-loaded liposomes prepared from P90G. Two magnifications are shown.

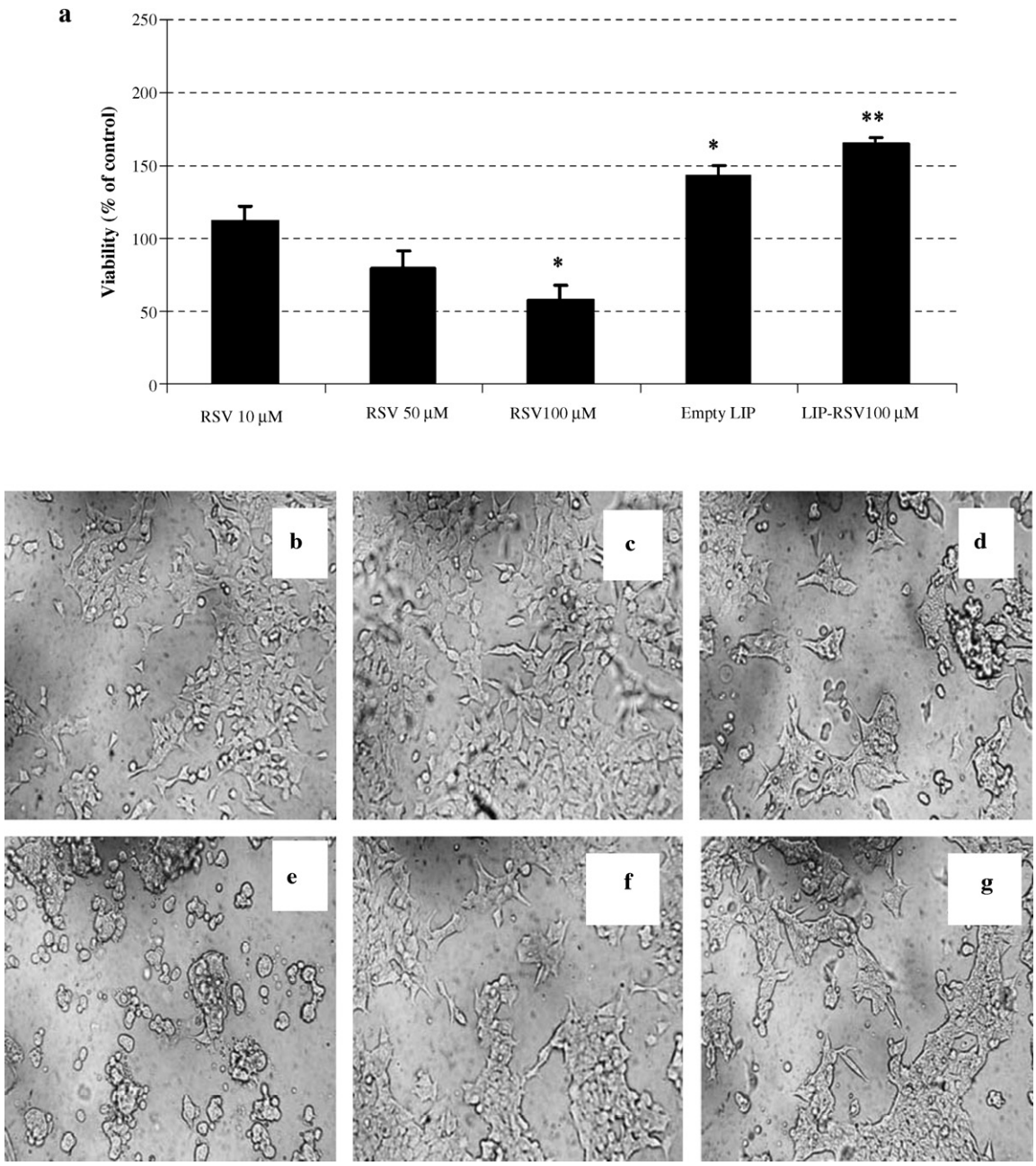

Fig. 3. (a) Influence of resveratrol (RSV) concentration and liposomal incorporation on the viability of HEK 293 cells. Values are means ± S.D. of five independent experiments, each performed in triplicate, $^{**}p < 0.001$, $^{*}p < 0.05$ difference from control, representing untreated cells. Morphological appearance of HEK 293 cells under the inverted light microscope (phase annulus of 10×); (b)untreated control cells; (c)–(e) cells treated with 10, 50 and 100 μM resveratrol, respectively; (f) cells treated with empty liposomes; (g) cells treated with 100 μM resveratrol incorporated in liposomes. Micrographs were taken 24 h after incubation with the samples.

untreated control cells (Fig. 3b), and assumed a rounded shape and showed cell blending (Fig. 3e). Moreover, the cell shape modification, the progressive detachment and the appearance of cell clusters induced by resveratrol were all dose-dependent. Still at 50 μM resveratrol showed the cytotoxic effect (Fig. 3d), while at 10 μM (Fig. 3c) resveratrol did not affect the morphology of HEK 293 cells, which appeared as a confluent monolayer of flat, polygonal, adherent cells similar to control cells (Fig. 3b), and cell number was clearly increased.

Low concentrations of resveratrol thus did not impair cell viability, and its proliferative effect was inversely proportional to its concentration.

3.3. Enhancement of resveratrol efficacy by liposomal incorporation

The activity of resveratrol when incorporated into liposomes was investigated. MTS results provided strong evidence that resveratrol formulated in liposomes significantly increased cell proliferation, up to 165% of untreated cells (Fig. 3a). No significant influence of liposomal composition (type of phospholipid used) or of the loaded dose of resveratrol (10 or 100 μM) was observed, and notably, the incorporation of resveratrol in liposomes eliminates its cytotoxicity at 100 μM. This is probably due to slower uptake of liposomal resveratrol, compared to the free drug, by the cells result-

ing from slow drug release by the carrier, thus avoiding immediate and massive intracellular concentrations.

Under the inverted light microscope, increased numbers of cells and normal morphological appearance were evident (Fig. 3f and g; cf. b), which showed the biocompatibility and non-toxicity of the liposomes and confirmed the elimination of the cytotoxicity of resveratrol at high concentrations (compare Fig. 3g and e).

The increase of cell viability appeared to be due first to the liposomes themselves, since cell activation occurred even after treatment with empty liposomes, and secondly to the release of resveratrol from the carrier. For cytoplasmic delivery, liposomes have long been used to deliver drugs, based on the assumption that liposomes membranes fuse with cell membranes or that the liposomes are endocytosed by the cells and then release their content into the cytoplasm. The mechanism of uptake is dependent on the particular liposome formulation used, i.e., a negative surface charge improves endocytosis. Once liposomes have interacted with cells, the stimulation of proliferation may be due to several factors. Liposomes may lead to an enhancement of the uptake of extracellular nutrients and *in situ* encapsulation (and thus isolation) of waste products of cell metabolism. Imagawa et al. (1989) found that exogenous phospholipids, added as liposomes, stimulate proliferation in mouse mammary epithelial cells by acting as mitogens. Given the fact that lipids play at least two roles in cellular regulation – a structural role as the building blocks of membranes and a role in transducing intra- or extracellular signals – they suggested that phospholipid turnover in cells can result in the activation of multiple growth-

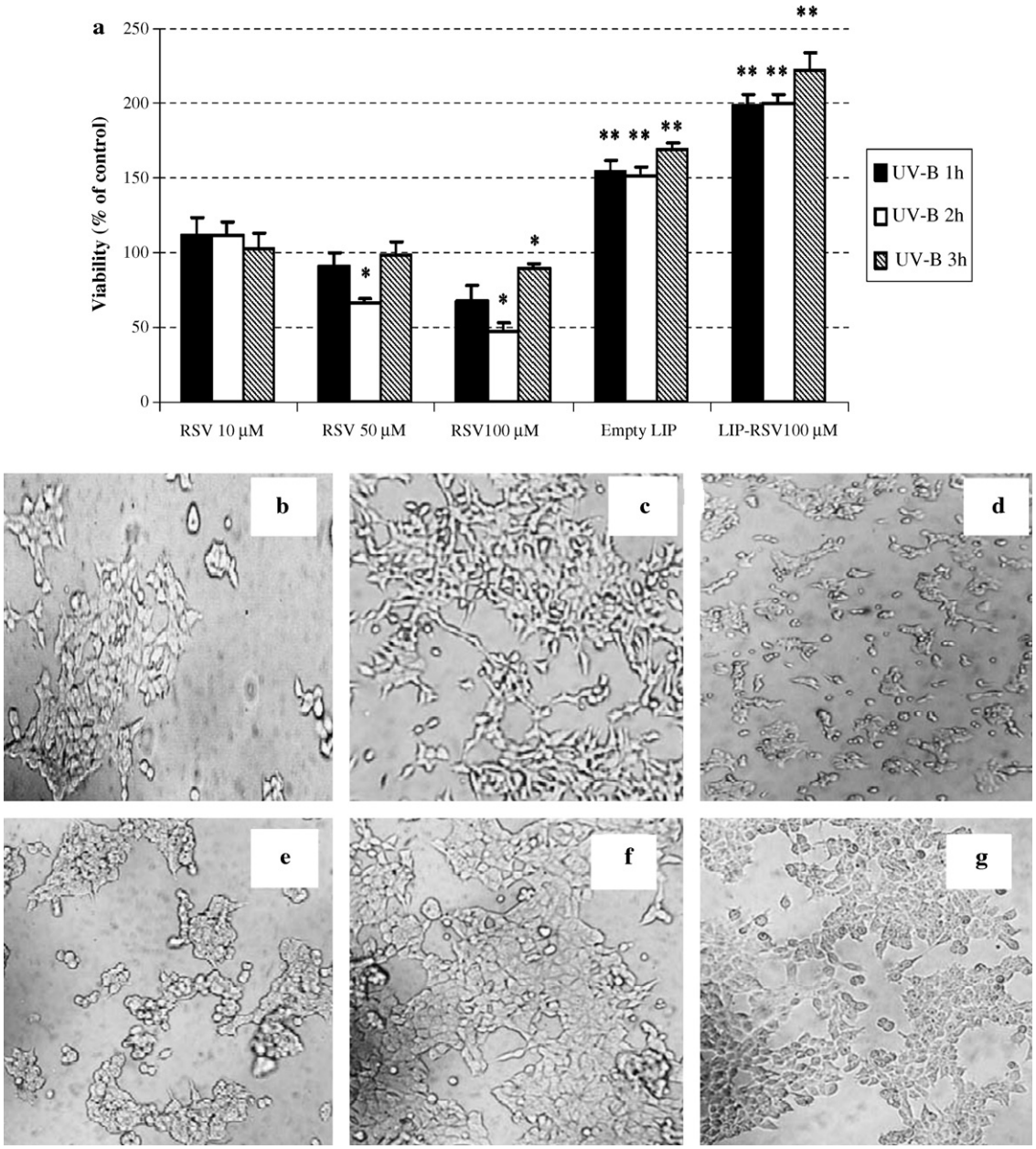

Fig. 4. (a) Effect of free and liposomal resveratrol (RSV) on the viability of HEK 293 cells after 1, 2 or 3 h of UV-B irradiation (each hour corresponding to 1.26 J). Data are expressed as means ± S.D. of five independent experiments, each performed in triplicate, $^{**}p < 0.001$, $^{*}p < 0.05$ difference from control, representing untreated but irradiated cells. Morphological appearance of HEK 293 cells after 1 h UV-B irradiation under the inverted light microscope (phase annulus of 10×); (b) untreated control cells; (c)–(g), cells treated with 10, 50, 100 μM resveratrol, empty liposomes and liposomes with 100 μM of resveratrol. Micrographs were taken after 24 h incubation with the test samples.

regulatory pathways and that phospholipid metabolism plays a crucial role in transducing growth-regulatory signals. Moreover, Schmidt et al. (1991) evidenced the positive influence of empty liposomes on cultured human epidermal keratinocytes. Small unilamellar dipalmitoylphosphatidylcholine vesicles caused a greater than twofold increase in cellular cholesterol synthesis, as if the cells were trying to maintain the original cholesterol/phospholipid ratio. Furthermore, the addition of cholesterol-enriched liposomes to keratinocytes treated with inhibitors of cholesterol synthesis revealed an increase of the total cellular cholesterol due to the incorporation of vesicular cholesterol. These findings point to the fact that liposomes themselves can regulate cell differentiation and thus may play a role other than drug carrier in the treatment of skin diseases, thereby contributing to the effect of resveratrol.

3.4. Evaluation of the cell proliferative response to resveratrol under stress conditions

The effect of different time UV-B radiation on cell viability was determined. UV-B radiation acts as a stress factor reducing viability of control cells for two times in 1 h. The protection contribution of resveratrol presence to the radiation consequences is presented in Fig. 4. It is shown that resveratrol at 100 μM suppressed cell proliferation, indicating that it was ineffective in protecting HEK 293 cells against UV-B damage. On the contrary, an increase in cell growth was observed at lower concentrations of resveratrol (50 and 10 μM), resveratrol at 10 μM providing photoprotection against up to 3 h irradiation (Fig. 4a). These results are in agreement with the findings of Howitz et al. (2003) who observed that the photoprotective effect of resveratrol from radiation-induced apoptosis, in HEK 293 cells, was reversed at concentrations greater than 50 μM.

Light micrographs show the morphological features of irradiated cells and provide visual confirmation of the MTS results (Fig. 4b–g). Comparing UV-B-irradiated and non-irradiated cells, it is evident the deleterious effect of UV-B light, which induced a dramatic decline in control cell numbers and round shaped and still adherent cells can be seen (Figs. 3b and 4b). Prior treatment with resveratrol at 100 μM affected cell viability again, due to the toxic effect of the drug observed also on non-irradiated cells (Fig. 3e), since rounded, suspended cells and aggregates of cell debris appeared (Fig. 4e). Similar drastic changes were seen in cells pretreated with resveratrol at 50 μM (Fig. 4d). On the contrary, in cultured cells pretreated with resveratrol at 10 μM, no morphological modifications or apoptotic features were apparent. Cells remained intact, adherent and were not reduced in number (Fig. 4c). Taken together these results confirm that a low concentration of resveratrol (10 μM) protects against the harmful effects of UV-B.

We additionally evaluated the effect of pretreating cells with resveratrol-loaded liposomes prior to UV-B irradiation. Empty liposomes were also tested to ascertain the effect of the carrier itself. An increase in cell growth was measured for empty liposomes, up to 170% of control (untreated irradiated cells) after 3 h of UV-B exposure (Fig. 4a), evidencing the photoprotective effect of the carrier. In the presence of resveratrol-loaded liposomes, the cell viability was further increased, up to 223% of control (after 3 h of irradiation), due

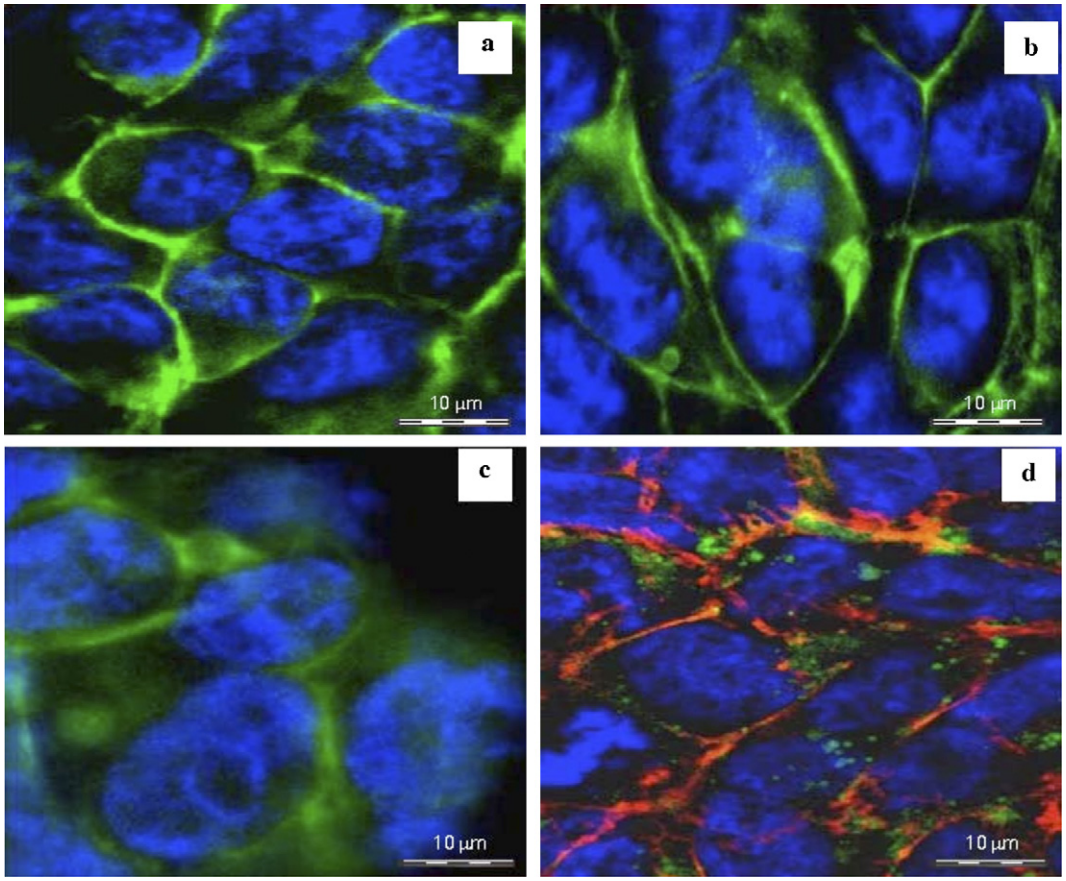

Fig. 5. Fluorescence micrographs showing the morphological changes induced by resveratrol in HEK 293 cells with blue-stained nuclei and green-stained actin cytoskeleton. (a) Untreated cells; (b) cells treated with 10 μM resveratrol; (c) with 100 μM resveratrol; (d) cells treated with 100 μM resveratrol incorporated into green-fluorescent liposomes have blue-stained nuclei and red-stained actin cytoskeleton. Any explicit morphological change is evident in micrographs (b) and (d) whereas, a blurred green fluorescence of actin, indicating a loss of cell membrane integrity, is apparent in micrograph (c).

to the synergistic effect of liposomes and resveratrol. No significant influence of the liposomal lipid composition (formulations A and B) or of the resveratrol-loaded dose (10 or 100 μM) was observed. Photomicrographs provided visual evidence of the marked stimulation of cell growth and the absence of any detectable structural alteration of cells induced by UV-B exposure (Fig. 4f and g). These data indicate that liposomes promote cell proliferation even more under the stress caused by UV-B light, and increase the ability of cells to survive and cope with the oxidative stress. This stimulatory effect increases as soon as resveratrol is released by the carrier and is thus able to exert its antioxidant action by scavenging ROS and inhibiting lipid peroxidation, as suggested in previous publications (Afaq et al., 2003; Howitz et al., 2003).

3.5. Effect of resveratrol on HEK 293 cell structure alteration investigated by fluorescence microscopy

The effect of resveratrol on the morphology of HEK 293 cells was examined by fluorescence microscopy. This technique allowed us to observe any structural alteration occurring to the cells and to elucidate fine details not detectable by the inverted light microscope.

Fluorescence micrographs clearly revealed the dose-dependent stimulatory effect of resveratrol on HEK 293 cells. At low concentration (10 μM) resveratrol increased the number of cells without any evidence of toxicity (Fig. 5a and b). In contrast, cells treated with 100 μM resveratrol showed a loss of membrane integrity, due to polymerized actin degradation, impairing the normal functional status of the cells (Fig. 5c), as demonstrated by the MTS data.

The interaction of the liposomes with HEK 293 cells, as well as their intracellular fate, was also studied by fluorescence microscopy. Cell proliferation was promoted by treatment with both empty and resveratrol-loaded liposomes (10 and 100 μM resveratrol). No influence of liposomal lipid composition (formulations A and B) was observed. No signs of cytotoxicity were shown on the cells treated with liposomes containing 100 μM resveratrol (Fig. 5d). The intense bright green intracellular punctate fluorescence indicates internalization of the liposomes and their distribution within the cytoplasm.

In addition, UV-B radiation was observed, on the green-fluorescent stained actin fibers, to cause photooxidative damage to HEK 293 cells (Fig. 6a). A prior treatment with 10 μM resveratrol provided slight protection against UV-B radiation (Fig. 6b), presumably by its antioxidant activity, preventing cell membrane degradation and loss of cell functionality. Increasing the concentration of resveratrol up to 100 μM, resulted in a large diminution of actin green fluorescence due to extensive cell membrane disruption and disorganization, as well as nuclear degradation indicative of apparent apoptotic cell death, confirming the cytotoxicity of high concentrations of resveratrol and its inability to protect the cells against radiation (Fig. 6c). This results well correlate with obtained viability data (Fig. 4a).

Superior UV-B protection of irradiated HEK 293 cells was achieved by incorporating resveratrol in liposomes. The images

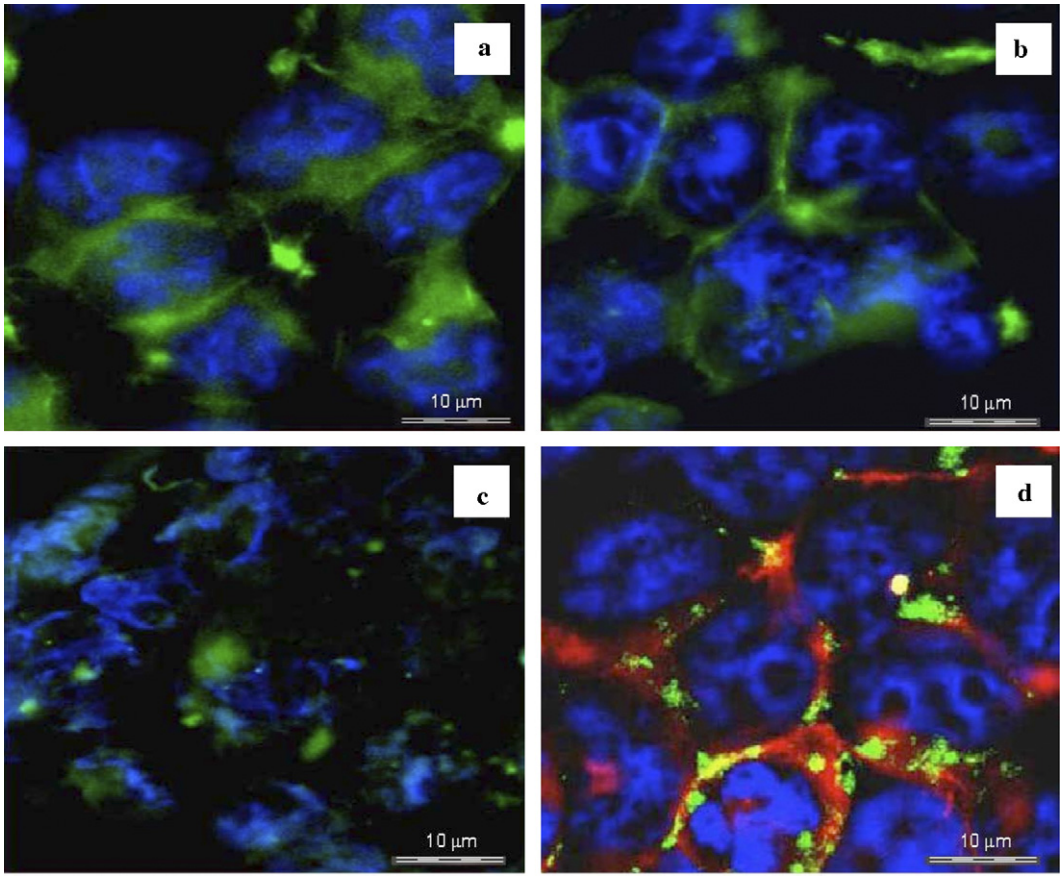

Fig. 6. Morphological changes induced by exposure of HEK 293 cells to UV-B light (1 h irradiation corresponding to 1.26 J) and protective effect provided by resveratrol. Fluorescence microscopy micrographs of cells identified with blue-stained nuclei and green-stained actin cytoskeleton. (a) Untreated cells; (b) cells treated with 10 μM resveratrol; (c) with 100 μM resveratrol; (d) cells treated with 100 μM resveratrol incorporated in green-fluorescent liposomes (blue-stained nuclei and red-stained actin cytoskeleton). Cell protection against radiation offered by 10 μM resveratrol and liposomes is shown in micrographs (b) and (d). Combined damaging effect of radiation and 100 μM resveratrol, causing membrane disruption and nuclear disbanding, appearing as dispelled blue colour of DNA intercalating dye, is clearly visible in micrograph (c).

show, besides the localization of resveratrol-loaded coumarin-labelled liposomes (green-fluorescent dots) on the surface of the cell membrane and predominantly in the cytoplasm, that cells are healthy and increased in number. The effect was independent of the concentration of resveratrol (10 and 100 μM) in the liposomes and of the formulation tested (A and B) (Fig. 6d). Thus, resveratrol in liposomes was much more effective than free resveratrol at 10 μM against UV-B-induced oxidative damage. Altogether these findings emphasize that liposomes protect the cells against the cytotoxicity of high concentration of resveratrol and significantly increase its UV-B protective activity.

4. Conclusions

Resveratrol has been shown to exert marked dose-dependent proliferative and photoprotective effects on HEK 293 cells. The enhanced effect observed when it is incorporated into liposomes suggest the use of the latter as a potent tool in the entrapment and delivery of resveratrol to cells. This discovery shows that liposomal incorporation may be a novel and promising strategy to enhance the efficacy of resveratrol in the prevention and treatment of human skin disorders caused by the excessive exposure to UV radiation.

Acknowledgements

We acknowledge Prof. Roger H. Pain for proof reading the manuscript. This work was funded by EU under the Marie Curie Early Stage Scholarship Program, Project name: Towards a Euro-PhD in advanced drug delivery, Contract No.: MEST-CT-2004-504992.

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