Original Communication

Liposomes as carriers of resveratrol and vitamin E: Evaluating ameliorative antioxidant effect using chemical and cellular test systems

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Abstract: Resveratrol (RES) in combination with antioxidant vitamins is reported to be more effective in protecting the cells from oxidative stress rather than any of these antioxidants alone. In continuation to our previous work using resveratrol and vitamin C, our main aim was to evaluate the antioxidant restorative effect using chemical and cellular test systems on resveratrol co-encapsulated vitamin E (VE) within liposomes. Z-average size was less than 135 nm, polydispersity index < 0.3; zeta potential > than ± 30 mV and encapsulation efficiency of RES and VE > 90% and 79% respectively. Chemiluminescence measurement indicated that the antioxidative activity of RES could be increased when VE was additionally loaded into liposomes. Inhibition of AAPH induced luminol enhanced chemiluminescence displayed 90% improvement ($P < 0.001$) in comparison to control; on the other hand 70% luminescence inhibition of ROS production in isolated blood leukocytes ($P < 0.001$) was observed. Intracellular oxygen-derived radicals measured by flow cytometry using 2'-7'-dichlorodihydrofluorescein diacetate demonstrated about 1.7 fold ($P < 0.05$) and 1.5 fold ($P < 0.001$) enhancement of radical scavenging activity in buffy coats under basal conditions and human umbilical vein endothelial cells after stimulation by H_2O_2 respectively. The cellular systems evidenced the ability of liposome loaded antioxidants to scavenge ROS in the extra and intracellular space, confirming enhanced antioxidative effectivity of RES in the presence of VE, which did not occur in combination with vitamin C. Hence it might be possible to improve the antioxidative effectivity of RES by other/additional antioxidants.

Keywords: resveratrol, vitamin E, antioxidants, luminol enhanced chemiluminescence, flow cytometry

Abbreviations

Resveratrol (RES); reactive oxygen species (ROS); vitamin C (VC); vitamin E (VE); LR – resveratrol loaded liposomes; LRVE – resveratrol loaded liposomes with vitamin E; resveratrol standard (RES-std); phospholipon 90H (PL); cholesterol (CH), dicetyl phosphate (DP) High performance liquid chromatography (HPLC); percentage encapsulation efficiency % EE); polydispersity index (PDI);luminol-enhanced chemiluminescence (LEC); 2, 2'-azobis [2-amidinopropane dihydrochloride] (AAPH); 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA); 2'-7'-dichlorodihydrofluorescein (DCF);human umbilical vein endothelial cells (HUVECs);Fluorescence activated cell sorting (FACS) hydrogen peroxide (H_2O_2); 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT);standard error of mean (SEM); analysis of variance (ANOVA); Nicotinamide adenine dinucleotide phosphate (NADPH); d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS).

Introduction

Resveratrol (RES) is a naturally occurring stilbene present in many plant species including several used in human nutrition such as grapes, raspberry juice or wine, peanuts, plums etc., [1]. The beneficial effect of red wine (or Mediterranean diet) is at least in part attributed to RES, yet a wide variety of therapeutic effects are clearly demonstrated: anti-cancer, anti-inflammation, anti-aging, cardioprotection, anti-atherosclerosis and many more which are related to its

antioxidative property, i.e. the ability to prevent oxidative stress [2, 3].

Oxidative stress, an increased cellular production of reactive oxygen species (ROS) is a general mechanism characteristic for inflammation, but also recognized in the last years, as a transient physiological mechanism involved in different cellular signaling pathways [2]. The resulting long lasting disturbance of pro-oxidant and antioxidant balance in favor of the oxidant species may cause pathological effects [4–6].

Many studies in experimental systems revealed different mechanisms for the antioxidative protection by resveratrol, including effects on the level of gene expression as well as on the level of enzyme activity [7]. In addition, due to the hydroxyl groups bound to the stilbene skeleton, a direct scavenging effect is possible by chemical reaction with ROS [2]. Widespread protective role of resveratrol has been extensively reported [3].

However, contradictory results were seen in many clinical trials with antioxidants in general, especially also with RES, hence a disappointing therapeutical outcome has been observed [8, 9]. Frombaum et al. 2012 reported that one reason for therapeutic failure of RES is limited bioavailability after oral administration [6].

To improve the effectiveness of antioxidants and overcome the solubility limitation, their coupling to affinity carriers such as liposomes could offer chemical and biological protection [10–12]. With the possibility to encapsulate more than one antioxidant in the vesicular system, liposomes could be more beneficial in prolonging their circulation times, coordinating their release into the body and ameliorating oxidant-induced tissue injuries [13].

Combination of resveratrol with other antioxidants has been reported to be more effective in protecting the cells from oxidative stress instead of using these antioxidants alone [14, 15]. E.g. resveratrol in combination of vitamin E has been evaluated for brain targeting in the treatment of Parkinson's disease in form of nanoemulsion [16]. In our previous work we demonstrated that there was no additive antioxidative effect, when RES-containing liposomes were delivered in presence of vitamin C (VC), a water-soluble antioxidant [17].

Here we report the next attempt to evaluate the antioxidant restorative effect of resveratrol co-delivered via liposomes together with vitamin E (VE) using different chemical and cellular systems.

Materials and methods

Materials

AAPH (2,2'-Azobis (2-amidinopropane) dihydrochloride was procured from Polyscience, Warrington, USA. Resveratrol, cholesterol, vitamin E, dihexadecyl phosphate, Triton-X 100, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Sigma-Aldrich, Steinheim, Germany. Zymosan A was obtained from Sigma, Deisenhofen, Germany. Phospholipon 90H (PL) was a kind gift sample from Lipoid AG, Cologne, Germany. Luminol, methanol, ethanol, chloroform, DMSO were obtained from Merck, Darmstadt, Germany. HPLC estimation of vitamin E was commercially analysed by SGS INSTITUT FRESENIUS GmbH Tegeler Weg 33, Berlin, Germany. Buffy coat cells were kindly provided by Institute of Tranfusion Medicine, Universität Tübingen, Germany. Primary cultures of Human umbilical vein endothelial cells (HUVECs), Cat No: C-12203 was obtained from Promocell, Germany.

Methods

Since all methods used here were already comprehensively described [17] only short descriptions are given here.

Formulation and physico-chemical characterization of liposomes

Resveratrol liposomes with and without VE were prepared as per the composition shown in Table 1 using thin film hydration method [18]. Briefly, lipid phase comprising of phospholipon 90H, cholesterol, dicetyl phosphate along with RES and VE were dissolved in chloroform: methanol (2:1 v/v) mixture in a dry round bottom flask. Organic solvents were removed using a vacuum evaporator (Rotavapor-R, W.Büchi, Flawil Schweiz) above the lipid transition temperature (51 °C) to obtain an uniform, thin lipid film. The deposited lipid film was hydrated with appropriate volume of water (double distilled) by rotation for 1 h at 51 °C. Small unilamellar (SUV) liposomes were further obtained by subjecting the dispersions to probe sonication (Ultrosonic Processor, UP200S, Hielscher Ultrasound Technology) for 2 to 6 min using cooling pads. Finally the liposomal dispersions prepared were stored at room temperature for 2 h to anneal any structural defects.

Liposomes comprised of RES with and without VE termed as LR – resveratrol loaded liposomes; LRVE – resveratrol loaded liposomes with vitamin E. Liposomes were prepared similarly without both the antioxidants – resveratrol and vitamin E termed as L-Blank. Standard drug stock solution of RES (200 μM) was prepared in methanol (RES-std), which was stored at −20 °C and used to compare antioxidative capacity of liposomal formulations.

Encapsulation efficiency (%) was determined after separation of free and entrapped drug using ultracentrifugation (Beckman L7 ultracentrifuge, Beckmann Coulter GmbH, Germany), further RES and VE content was determined directly from the liposomal pellet using HPLC.

Table 1. Formulation and characterization of resveratrol liposomes with and without vitamin E

Formulation code	Molar ratio PL: CH: DP	Resveratrol/ Vitamin E (μM)	Z average (nm)	Poly dispersity Index	Zeta potential (mV)	Encapsulation efficiency (%) Resveratrol	Vitamin E
LR1	2: 0.4: 0.6	100/0	133.0 ± 5.21	0.265 ± 0.01	−37.26 ± 1.16	78.53 ± 5.63	–
LR 2	3.4: 0.4: 0.6	150/0	131.8 ± 8.23	0.249 ± 0.01	−48.77 ± 0.44	80.11 ± 3.15	–
LR 3	4: 0.8: 1.2	200/0	134.2 ± 9.13	0.321 ± 0.01	−49.28 ± 0.03	85.19 ± 6.96	–
LRVE 1	2: 0.4: 0.6	100/100	126.3 ± 7.53	0.182 ± 0.03	−64.35 ± 0.16	90.31 ± 7.54	95.43 ± 5.23
LRVE 2	3.4: 0.4: 0.6	150/150	125.5 ± 9.25	0.184 ± 0.02	−53.40 ± 0.88	89.45 ± 4.87	94.12 ± 6.15
LRVE 3	4: 0.8: 1.2	200/200	126.3 ± 6.65	0.189 ± 0.04	−50.73 ± 0.29	85.59 ± 6.23	90.57 ± 4.74

Values are expressed as mean (n = 6) with standard deviation. PL – Phospholipon 90H; CH – cholesterol; DP –dicetyl phosphate; LR – liposomes loaded with resveratrol alone; LRVE – liposomes loaded with resveratrol and vitamin E.

$$\text{Encapsulation Efficiency} = \frac{\text{ERES}}{\text{TRES}} \times 100 \qquad [1]$$

where ERES was determined by taking the difference of total RES content and surface RES content; TRES – initial amount of RES added.

Vitamin E concentrations were commercially analyzed by SGS Institute Fresenius GmbH, Berlin, Germany, using the official collection of analytical methods (§ 64 LFGB-Method L – 4900-5 tocopherols and tocotrienols).

Particle size (Z-average mean size and polydispersity index) and ζ – potential were determined using a Nanosizer (Nano-ZS, Nanoseries, Malvern Instruments, UK) after appropriate dilution of liposome preparation. Morophological examination of the liposome preparations was carried out using freeze fracture electron microscopy [19].

Measurement of antioxidant activity based on luminol-enhanced chemiluminescence (LEC)

LEC was measured by two different test systems; in both, time-dependent luminescence was integrated and quantified (counts per time) by a luminometer (Berthold 9600, Wildbad, Germany). The first step consisted of spontaneous decay of the azo compound 2, 2'-azobis (2-amidinopropane dihydrochloride) (AAPH) producing peroxyl free radicals. Next, LEC values obtained during the reaction in the presence of the liposomal antioxidants were related to that of the antioxidant-free control reaction. The results are expressed as relative radical scavenging activity whereby 100% correspond to complete suppression radical generation.

In the second test system LEC was used to measure basal as well as zymosan-stimulated free radical secretion of isolated blood leukocytes. The inhibiting effects of the liposomal preparations were related to the corresponding control reactions and are given as relative luminescence inhibition (100% means complete inhibition of chemiluminescence) [17].

Measurement of antioxidant activity using fluorescence activated cell sorting (FACS)

Leukocytes were isolated as buffy coat cells from one healthy donor and incubated without antioxidants or together with the liposomal preparations for 60 min, and, after the addition of 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (final concentration 10 μM) for further 30 min. Then the cells were washed and the emission of cellularly trapped DCF, oxidized by basally generated oxygen radicals was analyzed with flow cytometry; FACS-calibur from Becton Dickinson; Heidelberg, Germany. Fluorescence intensity of DCF was measured in FL-1 with an excitation wavelength of 488 nm and emission wavelength of 530 nm.

Similarly, HUVECs cultured to confluence from early passages 3-6 were pre-incubated with H_2DCF-DA dye and sample formulations for 1 hr. After a change of the medium, the cells were further incubated for 30 min in the presence of H_2O_2 (50 μM). Cells incubated without antioxidants or liposomal preparations were defined as control.

Cytotoxicity of formulations was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay using buffy coats and HUVECs, test samples (LR3 and LRVE1 and pure resveratrol) were incubated at 37 °C (5% CO_2). After 24 h of incubation, 100 μL of fresh media and 10 μL MTT were added to each assay, absorbance read at 570 nm using VERSAmax plate reader (Molecular Devices VERSAmax Tunable Microplate Reader, California, USA).

Statistics

Data is expressed as mean ± SEM; n represents the number of independent experiments. All the data was tested for normality and variance homoscedasticity and were log e transformed when necessary. Differences were tested for significance using one way ANOVA-Tukey's multiple comparison test and Student t-test. $P < 0.05$ was considered to be significant.

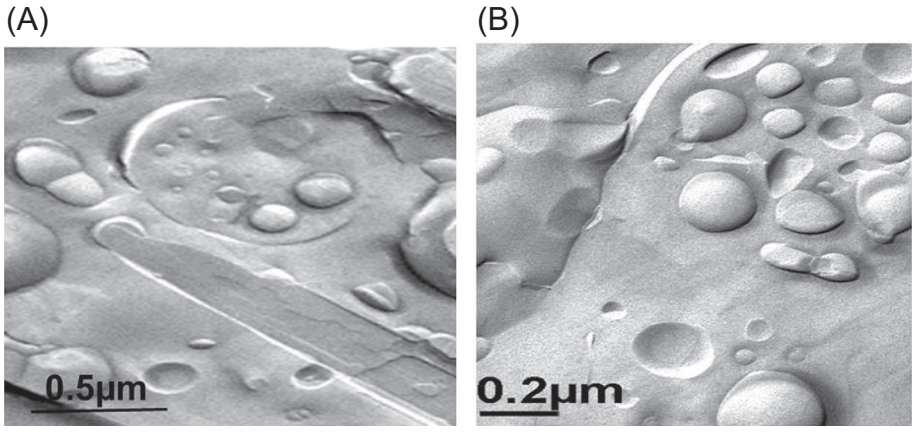

Figure 1. Freeze fracture electron micrographs of resveratrol liposomes. (A) Image depicts resveratrol liposomes (LR3), bar indicates 0.5 µm. (B) Image depicts resveratrol liposomes with vitamin E (LRVE1), bar indicates 0.2 µm.

Results and discussion

Liposomes were formulated by incorporating both the antioxidants: RES and VE in the lipid phase using thin film hydration method. The EE (%) of RES and VE in liposomal systems were analyzed after centrifugation and ranged from 78 to 90% for RES and 90 to 95% for VE, respectively (Table 1). These values are significantly higher than those for vitamin C [17] probably due to the fact that RES and VE are lipid soluble and are incorporated entirely in the bilayer. All the formulations exhibited an unimodal particle size distribution with PDI less than 0.3 and Z average size was in the range of 125 nm to 134 nm, further confirmed by freeze fracture electron micrographs (Figures 1A and 1B). As depicted in Table 1, a slight decrease in size of RES liposomes in presence of vitamin E (LRVE formulations) was observed, however difference was not significant ($P > 0.05$) when compared with LR formulations. Other authors have demonstrated that varying the sonication time from 1 to 3 min led to changes in the particle size when combination of trans-resveratrol liposomes and D-α-tocopherol were formulated [20]. Surface charge reported as zeta potential values was higher than ± 30 mV, enhancing electrostatic stabilization of liposomal suspension with the negative charge due to addition of dicetyl phosphate. Comparison of physico-chemical properties of liposomes loaded with RES alone and in combination of VE indicates that the vitamin does not induce alterations. Similarly, presence of vitamin C in RES liposomes did not influence the physicochemical properties when compared with RES liposomes alone [17]. This infers that liposomes could be a suitable carrier system to encapsulate either water soluble antioxidants in the aqueous phase or lipid soluble antioxidants probably included within the lipid bilayer of the liposome along with lipophilic resveratrol.

Enhanced antioxidative effects of VE on resveratrol-loaded liposomes were observed using chemical and cell based assays. Using the LEC test system (Figures 2A and 2B), formulations revealed an increased antioxidative capacity with increasing content of resveratrol in liposomes. Combining the lowest tested concentration of RES and VE (LRVE1-10 µM each) led to significant improvement on radical scavenging activity due to synergistic effect of RES and VE ($P < 0.001$). This effect was less pronounced with higher concentrations of pure RES ($P < 0.01$). In contrast, addition of vitamin C did not improve this property correspondingly in our previous investigation [17]. ROS produced by activated phagocytes that leads to an extracellular secretion of oxygen free radicals was effectively inhibited by liposomal RES and VE, with 70% luminescence inhibition in relative to control ($P < 0.001$). Original tracing of free radical production of zymosan stimulated buffy coats is illustrated in Figure 2C, which confirms that liposomes loaded with RES and presence of VE quenched the ROS produced by activated phagocytes more effectively in comparison to methanolic RES standard solution.

Scavenging ROS in the extra- as well as the intracellular space by liposomal RES in presence of VE signified the synergistic antioxidative effect. This was further confirmed by flow cytometric analysis using H_2DCF-DA, a cell permeable, non-fluorescent pre-cursor of DCF which is an intracellular probe for oxidative stress. Spontaneous free radical production in buffy coat cells (Figure 3A) generated by NADPH oxidase as well as H_2O_2 induced oxidative stress in HUVECs (Figure 3B) was measured. Decrease in ROS production (represented as % ROS positive cells) indicated by decrease in DCF fluorescence was observed when liposomal resveratrol was co-delivered with VE. Finally, FACS study (Figures 4A and 4B) demonstrates enhanced radical scavenging activity by 1.7 fold and 1.5 fold in buffy coats

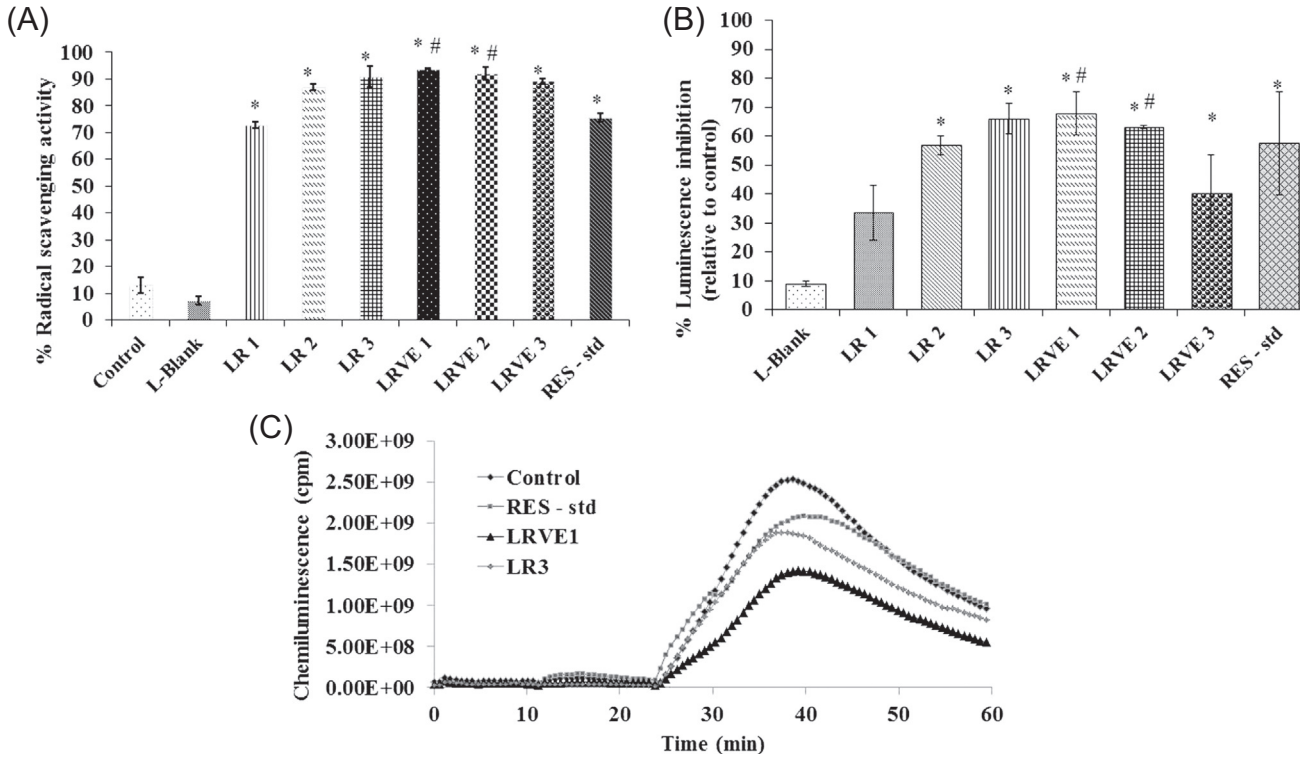

Figure 2. Antioxidative effect of liposomes loaded with resveratrol and addition of vitamin E using luminol-enhanced chemiluminescence. Values are expressed as mean (n = 6) and error bars represent standard error of mean. (A) Measurement of LEC reduction using AAPH reaction. *($P < 0.001$) indicates significant difference from control. #($P < 0.01$) indicates significant difference of LR3 and LRVE1 from RES-std. (B) Measurement of free radical inhibition in relative to control in buffy coats. *($P < 0.001$) indicates significant difference from control. #($P < 0.01$) indicates significant difference of LR3 and LRVE1 from RES-std. (C) Original tracing of antioxidative effects on free radical production of zymosan stimulated buffy coat cells and inhibition by various formulations. LR – liposomes loaded with resveratrol alone; LRVE – liposomes loaded with resveratrol and vitamin E; RES-std – Resveratrol standard in methanolic stock solution; control – pure methanolic solution with no antioxidants; L-blank – liposomes with no antioxidants.

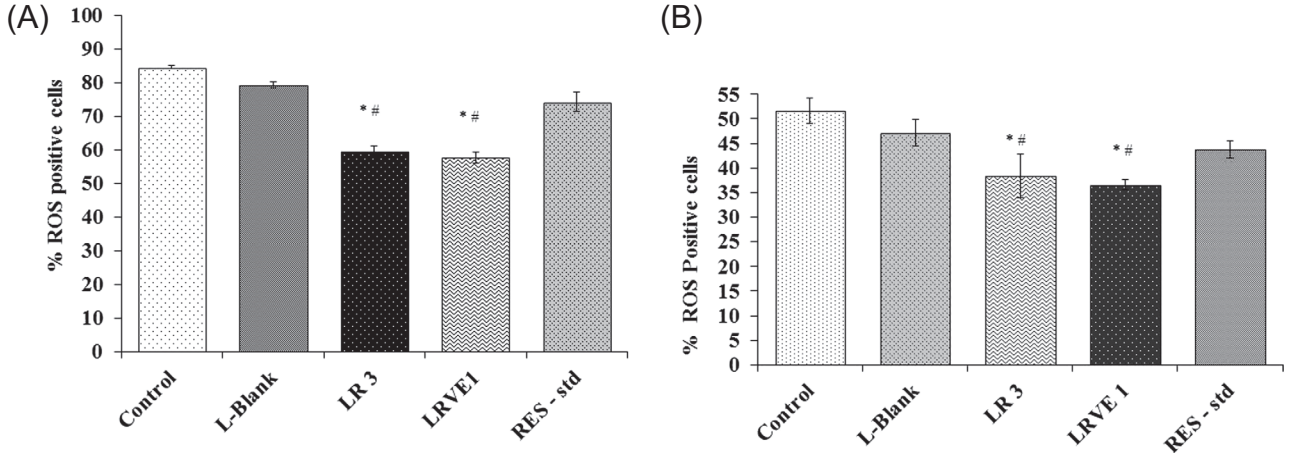

Figure 3. Measurement of ROS dependent DCF fluorescence using flow cytometry in presence of liposomes loaded with resveratrol and addition of vitamin E. Values are expressed as mean (n = 3) and error bars represent standard error of mean. (A) Antioxidative effect on spontaneous free radical production of buffy coats. *($P < 0.01$) indicates significant difference from respective control. #($P < 0.01$) indicates significant difference of LR3 and LRVE1 from RES-std. (B) Antioxidative effect in HUVECs. *($P < 0.1$) indicates significant difference from control. #($P < 0.5$) indicates significant difference from RES-std. LR – liposomes loaded with resveratrol alone; LRVE – liposomes loaded with resveratrol and vitamin E; RES-std – resveratrol standard in methanolic stock solution; control – pure methanolic solution with no antioxidants; L-blank – liposomes with no antioxidants.

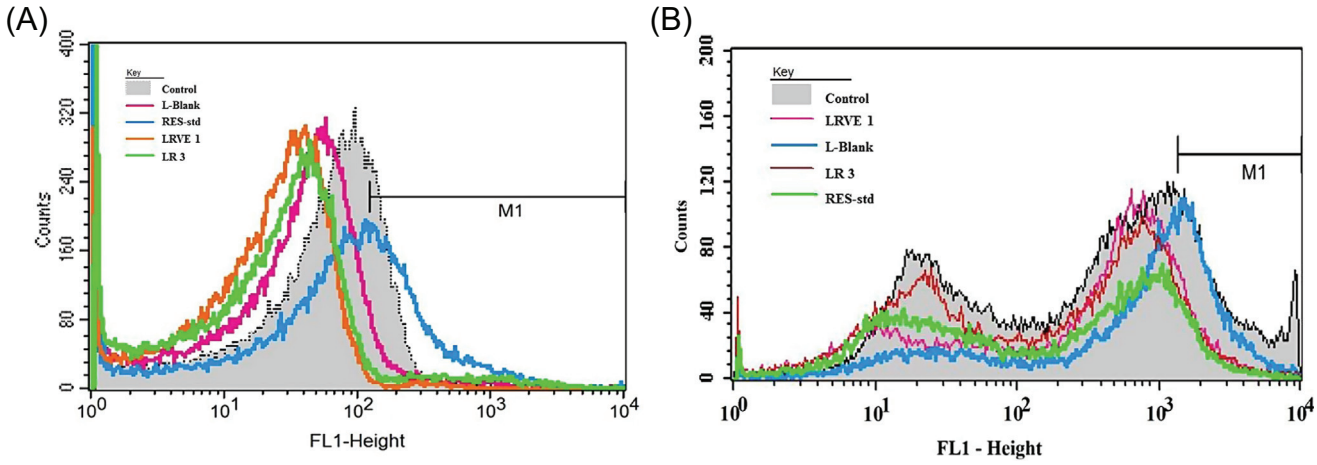

Figure 4. FACS Histogram overlay of liposomes loaded with resveratrol and vitamin E. (A) Measurement of spontaneous free radical production in buffy coats. *($P < 0.05$) indicates significant difference from control. #($P < 0.05$) indicates significant difference of LR3 and LRVE1 from RES-std. (B) Measurement of intracellular oxygen-derived radicals using HUVECs. *($P < 0.001$) indicates significant difference from control. #($P < 0.01$) indicates significant difference of LR3 and LRVE1 from RES-std. LR – liposomes loaded with resveratrol alone; LRVE – liposomes loaded with resveratrol and vitamin E; RES-std – resveratrol standard in methanolic stock solution; control – pure methanolic solution with no antioxidants; L-blank – liposomes with no antioxidants.

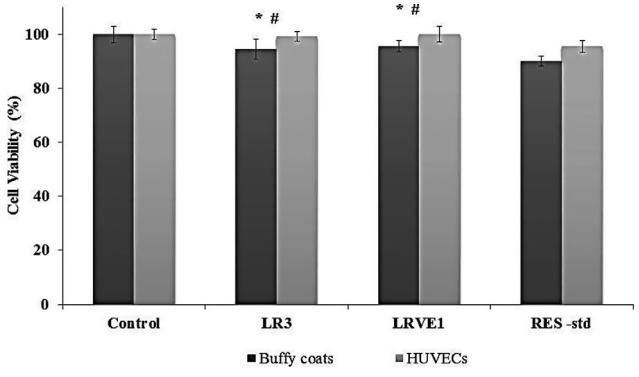

Figure 5. Cytotoxic effect of liposomes loaded with resveratrol and vitamin E and resveratrol standard in buffy coats and HUVECs. Values are expressed as mean (n = 3) and error bars represent standard error of mean. LR – liposomes loaded with resveratrol alone; LRVE – liposomes loaded with resveratrol and vitamin E; RES-std – resveratrol standard in methanolic stock solution; control – pure methanolic solution with no antioxidants.

($P < 0.05$) and HUVECs ($P < 0.01$) respectively at lowest tested concentrations of RES (10 μM) in LRVE1 in comparison to pure RES (20 μM). Previous studies with VC demonstrated that this antioxidant could not synergize this protection to a similar extent [17]. Absence of cytotoxicity of resveratrol standard (20 μM), liposomal RES (20 μM and 10 μM of resveratrol using LR3 and LRVE1 respectively) was confirmed using MTT tetrazolium assay and cell viability (%) was > 90% (Figure 5). Improvement of antioxidative properties of RES alone by loading into liposomes was also demonstrated in several other investigations [21, 22], as well as co-supplementation with hydrophilic or lipophilic additives [20, 23, 24]. Vijayakumar et al. reported that TPGS coated resveratrol liposomes were highly effective in the treatment of brain cancer [20], however TPGS is a water-soluble amphiphilic polymer unlike vitamin E which is lipophillic. On the other hand lipid nanoparticles containing resveratrol, vitamin E, and epigallocatechin gallate provided protection to the skin [23].

However, for the first time, our study demonstrates that in contrast to a water-soluble vitamin presence of a lipophilic vitamin could improve the antioxidant effect of RES when loaded into liposomes at a lower concentration (10 μM of RES in LRVE1) in comparison to RES liposomes (20 μM of RES in LR3).

Liposomes were successfully formulated encapsulating two lipophilic antioxidants in the lipid phase of the vesicle. FACS analysis demonstrated that the protection of oxidative injury within the intracellular compartments was pronounced when resveratrol was delivered using liposomes rather than methanolic solution of resveratrol. However, cell uptake studies and qualitative analysis using confocal microscopy could be performed to confirm the intracellular uptake of liposomes loaded with resveratrol with and without vitamin E.

Conclusion

Liposomal delivery of RES increases the antioxidative properties when compared to methanolic RES, yet, a further synergistic antioxidative effect was observed in presence of the lipid soluble vitamin E. Hence it might be possible to improve the antioxidative effectivity of RES by other/additional antioxidants.

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Conflict of interest
We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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