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In vitro protective effects of resveratrol against oxidative damage in human erythrocytes

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ABSTRACT

Resveratrol (RV) is a potent antioxidant, anticancer and anti-inflammatory agent. Its main target of action is the cell membrane; however, its effect on that of human erythrocytes has been scarcely investigated. With the aim to better understand the molecular mechanisms of the interaction of RV with cell membranes both human erythrocytes and molecular models of its membrane have been utilized. The latter consisted in bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the erythrocyte membrane, respectively. Results by X-ray diffraction showed that RV produced a significant structural perturbation on DMPC bilayers, but no effects were observed in DMPE. Scanning electron (SEM) and defocusing microscopy (DM) observations showed that RV induced morphological alterations to the red cells from the normal discoid shape to echinocytes. These results imply that RV was located in the outer monolayer of the erythrocyte membrane. Results of its antioxidant properties showed that RV neutralized the oxidative capacity of HClO on DMPC and DMPE bilayers. On the other hand, SEM and DM observations as well as hemolysis assays demonstrated the protective effect of RV against the deleterious effects of HClO upon human erythrocytes.

1. Introduction

Resveratrol (RV, trans-3,5,4′-trihydroxystilbene, Fig. 1) is a polyphenol non-flavonoid compound, particularly abundant in red grapes (*Vitis vinifera*) but it is also present in highly pigmented vegetables and fruits [1]. It has been proven to be a potent antioxidant [2], anticancer [3] and anti-inflammatory agent [4]. Functionally, RV belongs to phytoalexins, also called the plant antibiotics [5]. It exists as *cis*- and *trans*-isomers being the latter the preferred steric form, which is relatively stable if protected from high pH and light [6]. On the other hand, *cis*-resveratrol is not as biologically active as the *trans*- form, being the different molecular behavior of the two isomers dependent on their three-dimensional structure [7]. RV is considered to be one of the major constituents in red wine, which contains 6.5 mg/l resveratrol [8,9]. A primary impetus for research on RV was initiated from the observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red wine, a phenomenon known as the French paradox [5,6,10,11]. Several diseases like cancer, inflammation, cardiovascular disorders, rheumatoid arthritis and neurodegeneration have been shown to be related to excessive generation of reactive oxygen species (ROS), which can cause damage to crucial biomolecules such as nucleic acids, proteins, polyunsaturated fatty acids, carbohydrates and cell membranes [2,12–14]. RV possesses three phenolic groups and acts as a free radical scavenger by transferring the proton from its phenolic group to the free radicals [7,12]. The molecular mechanism of action of polyphenol antioxidants would depend on their structure as well as of their capacity to intercalate into cell membranes where they would interact with lipid bilayers, acting as radical scavengers and thus protecting the membrane from oxidative stress [5,12,15]. In spite of the health importance of RV and results showing that the main target of RV action is the cell membrane [1], its effect on cell membranes, particularly of the human erythrocytes, has been scarcely investigated [12]. Cell membrane is a diffusion barrier which protects the interior of the cell. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with chemical species.

With the aim to better understand the molecular mechanisms of the interaction of RV with cell membranes human erythrocytes and molecular models of its membrane were utilized. Human erythrocytes were chosen because of their only one membrane and no internal organelles which constitute an ideal cell system for studying interactions of chemical compounds with cell membranes [16]. On the other hand, although less

Abbreviations: RV, resveratrol; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; SEM, scanning electron microscopy; DM, defocusing microscopy

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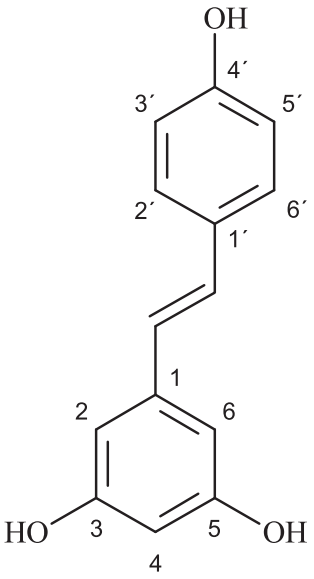

Fig. 1. Structural formula of resveratrol.

specialized than many other cell membranes they carry on enough functions in common with them such as active and passive transports, and the production of ionic and electric gradients to be considered representative of the plasma membrane in general. The molecular models of the erythrocyte membrane consisted in bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of cell membranes, particularly of the human erythrocyte, respectively [17,18]. The capacity of RV to perturb the bilayer structures of DMPC and DMPE was evaluated by X-ray diffraction; intact human erythrocytes were observed by scanning electron microscopy (SEM) and defocusing (DM) microscopy. These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other chemical compounds, particularly of native plant extracts [19–22]. The antioxidant properties of RV were evaluated in the molecular models of the erythrocyte membrane and human erythrocytes in vitro exposed to the oxidative stress induced by hypochlorous acid. HClO is a powerful natural oxidant that damages bacteria, endothelial cells, tumor cells and erythrocytes [23–26].

2. Materials and methods

2.1. Chemicals

Synthetic DMPC (lot 140PC-246, MW 677.9) and DMPE (lot 140PE-60, MW 635.9) from Sigma (AL. USA) and *trans*resveratrol (lot SZBA154XV, MW 228.2) from Fluka (Sigma, AL, USA) were used without further purification. Composition of phosphate buffered saline (PBS) was 150 mM NaCl, 1.9 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4. Concentration of HClO from commercial samples was spectrophotometrically determined at 292 nm ($\varepsilon = 350\ M^{-1}\ cm^{-1}$) [27].

2.2. X-ray diffraction studies of DMPC and DMPE multilayers

The capacity of RV to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. About 2 mg of each phospholipid was introduced into Eppendorf tubes which were then filled with 180 l of (a) distilled water (control), and (b) RV aqueous solutions in a range of concentrations (0.025–2.0 mM for DMPC and 1.0–10.0 mM for DMPE experiments). The specimens were shaken, incubated for 15 min at 30 °C and 60 °C with DMPC and DMPE, respectively and centrifuged for 20 min at 2500 rpm. Samples were then transferred to 1.5 mm diameter special glass capillaries (Glas-Technik&Konstruktion, Berlin, Germany) and X-ray diffracted utilizing Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray system. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. The relative reflection intensities were obtained in an MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 18 ± 1 °C, which is below the main phase transition temperature of both DMPC and DMPE. Higher temperatures would have induced transitions onto fluid phases making the detection of structural changes harder. Each experiment was performed in triplicate.

2.3. Scanning electron microscopy (SEM) studies on human erythrocytes

Five blood drops (100 μl) from a human healthy donor not receiving any pharmacological treatment were obtained by puncture of the ear lobule and received in an Eppendorff tube containing 100 μl of heparin (5000 UI/ml) in 900 μl of phosphate buffer saline (PBS), pH 7.4. Red blood cells were then centrifuged (1000 rpm × 10 min),washed three times in PBS, resuspended in PBS containing RV in a range of concentrations and then incubated at 37 °C for 1 h, period in line with the larger effects induced by compounds on red cell shape [28,29]. Controls were cells resuspended in PBS without RV. Specimens were fixed overnight at 4 °C by adding one drop of each sample to plastic tubes containing 500 μl of 2.5% glutaraldehyde in distilled water, reaching a final fixation concentration of about 2.4%. Samples were washed twice in distilled water and centrifuged (1000 rpm × 10 min.); about 20 μl of each sample were placed on siliconized Al glass covered stubs, air-dried at room temperature, gold coated for 3 min at 13.3 Pa in a sputter device (Edwards S 150, Sussex, England, and examined in a scanning electron microscope (JEOL JSM-6380LV, Japan)). Data were expressed as mean ± standard deviation of 50 cell counts.

2.4. Optical and defocusing microscopy (DM) studies of human erythrocytes

Erythrocyte shapes were visualized and then analyzed through three dimensional reconstructions using defocusing microscopy (DM). DM is an optical technique based on the visualization and analysis of contrast images of transparent objects seen out of focus using a bright field microscope. Red blood cells (RBC) were obtained from a healthy donor under no pharmacological treatment. Cells were centrifuged (1000 rpm for 10 min) and washed three times with PBS pH 7.4 with 1 mg/ml of bovine serum albumin (BSA). RBC solution was prepared diluting the washed blood 20 times in a solution of PBS and BSA. RV solution was prepared in the same preparation of PBS and BSA. In order to carry out the analysis, 1.7 ml of RBC diluted solution was placed in an acrylic cuvette, and visualized at the optical microscope. After that, a morphologically normal erythrocyte was selected by using software [30] and the concentration of RV was increased. In order to make three-dimensional reconstructions, two images were captured in the defocus positions +1 and −1 μm at 20 μM of RV with or without HClO in the RBC solution. The contrast of the images was obtained and three-dimensional shape reconstruction was directly calculated [30,31].

2.5. Hemolysis assays

Red blood cells (RBC) were obtained from a healthy consenting donor. 10 ml of heparinized blood was centrifuged (EYDAM, Germany) at 2500 rpm for 10 min. After removal of plasma and buffy coat, the RBC were washed three times with PBS at room temperature, and resuspended in PBS three times its volume for subsequent analyses [32]. RBC (10% v/v) were incubated in a shaking bath for 15 min. at 37 °C in PBS in the presence of increasing concentrations of RV in a final 1.5 ml volume. After cooling, increasing concentrations of HClO in PBS were added and

centrifuged at 2500 rpm for 5 min. Hemolysis was spectrophotometrically evaluated (Jasco, Japan) at 540 nm as hemoglobin (Hb) released from cells in the supernatant [33]. Results were related to control (100% hemolysis induced by 2.5 mM HClO on RBC).

3. Results

3.1. X-ray diffraction studies of DMPC and DMPE multilayers

Fig. 2A exhibits results obtained by incubating DMPC with water and RV. As expected, water altered the structure of DMPC as its bilayer repeat (phospholipid bilayer width plus the layer of water) increased from about 55 Å in its dry crystalline form [34] to 65 Å when immersed in water, and its small-angle reflections, which corresponds to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer width. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. These results were indicative of the $P_{\beta'}$ gel phase reached by DMPC bilayers. Fig. 2A discloses that after being exposed to increasing RV concentrations there was a gradual weakening of the low- and particularly of wide-4.2 Å lipid reflection intensities (indicated as SA and WA in the figure, respectively) which at 2 mM RV the 4.2 Å practically disappeared. From these results it can be concluded that RV produced a significant structural perturbation on the DMPC bilayer arrangement, being this effect somewhat higher in the hydrophobic region of DMPC. Fig. 2B shows the results of the X-ray diffraction analysis of DMPE bilayers incubated with water and RV. As reported elsewhere, water did not significantly affect the bilayer structure of DMPE [34]. Fig. 2B shows that increasing concentrations of RV did not cause any significant weakening of DMPE reflection intensities, all of which still remained practically unchanged even with 10 mM RV. Fig. 3 presents the protective capacity of RV on the oxidative property of HClO in DMPC and DMPE bilayers. Fig. 3A shows that from 100 mM to 350 mM HClO concentration there is an increasing perturbation of DMPC bilayer structure. However, as it can be appreciated in Fig. 3B, RV in the 2 to 7 mM concentration neutralized the deleterious effect of 250 mM HClO. The same experiments performed in DMPE bilayers show that 5 mM HClO induced a deep perturbation to DMPE bilayer structure (Fig. 3C), a concentration considerably lower than that observed in the case of DMPC. The fact that DMPE reflections almost disappeared indicated that the periodic structure of its multilamellar stacking was considerably lost; however, this effect was gradually reversed by increasing RV concentrations until completely neutralized by 5 mM RV (Fig. 3D). As a pale yellow color was observed in the aqueous mixtures, it is very likely that some interaction between RV and HClO also took place.

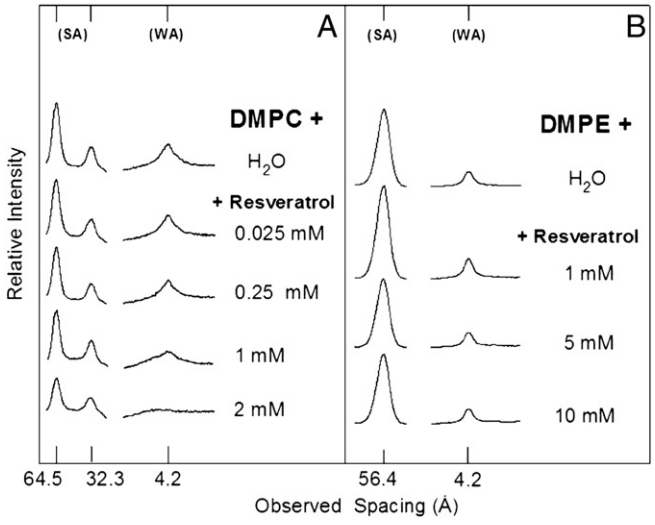

Fig. 2. X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE) in water and resveratrol; (SA) small-angle and (WA) wide-angle reflections.

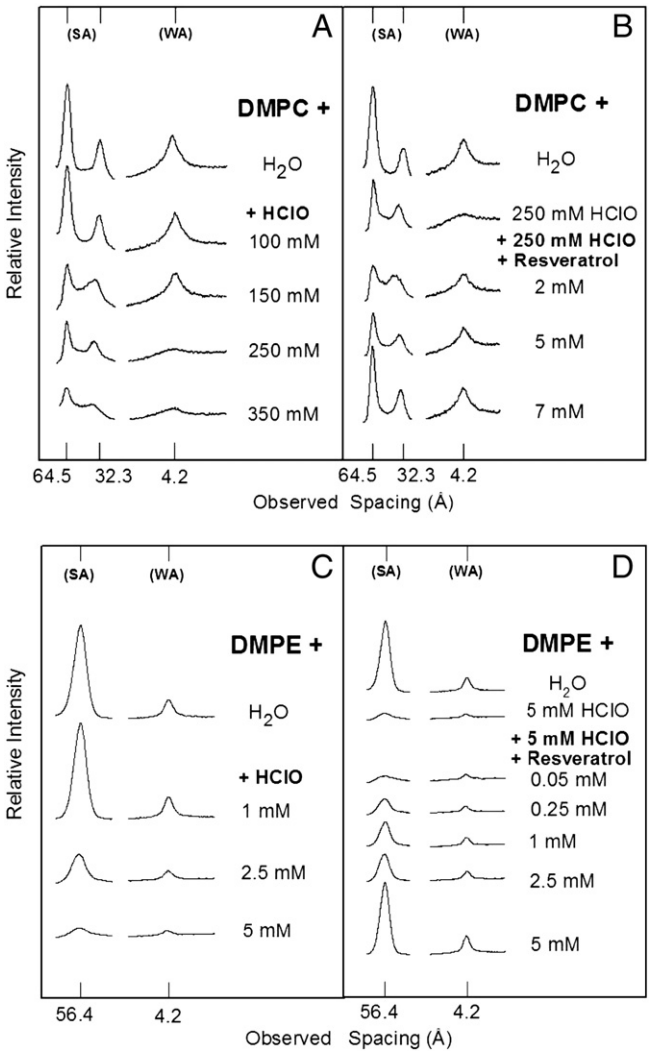

Fig. 3. X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) in water and HClO; (B) dimyristoylphosphatidylcholine (DMPC) in water, resveratrol and HClO; (C) dimyristoylphosphatidylethanolamine (DMPE) in water, and HClO; and (D) dimyristoylphosphatidylethanolamine (DMPE) in water, resveratrol and HClO; (SA) small-angle and (WA) wide-angle reflections.

3.2. Scanning electron microscopy (SEM) studies of human erythrocytes

SEM examinations of human erythrocytes incubated with RV in the 0.5 mM to 2 mM range indicated that RV induced echinocytosis. In that altered condition, red blood cells lost their normal biconcave profile (Fig. 4A) and presented a spiny configuration with blebs in their surfaces (Figs. 4B–D). The extent of these changes was dependent on the RV concentration. Fig. 5 presents the protective capacity of RV on the oxidative property of HClO in human erythrocytes. Fig. 5B shows that 0.5 mM HClO induced the formation of stomatocytes. However, this shape alteration of the red blood cells was reversed in samples previously incubated with RV. In fact, cells incubated with 0.25 mM RV and 0.5 mM HClO still show a large number of stomatocytes (Fig. 5C), but it can also be seen a few normocytes, echinocytes and knizocytes (cells with two or three concavities due to indentations in the cell membrane) (Fig. 5C).

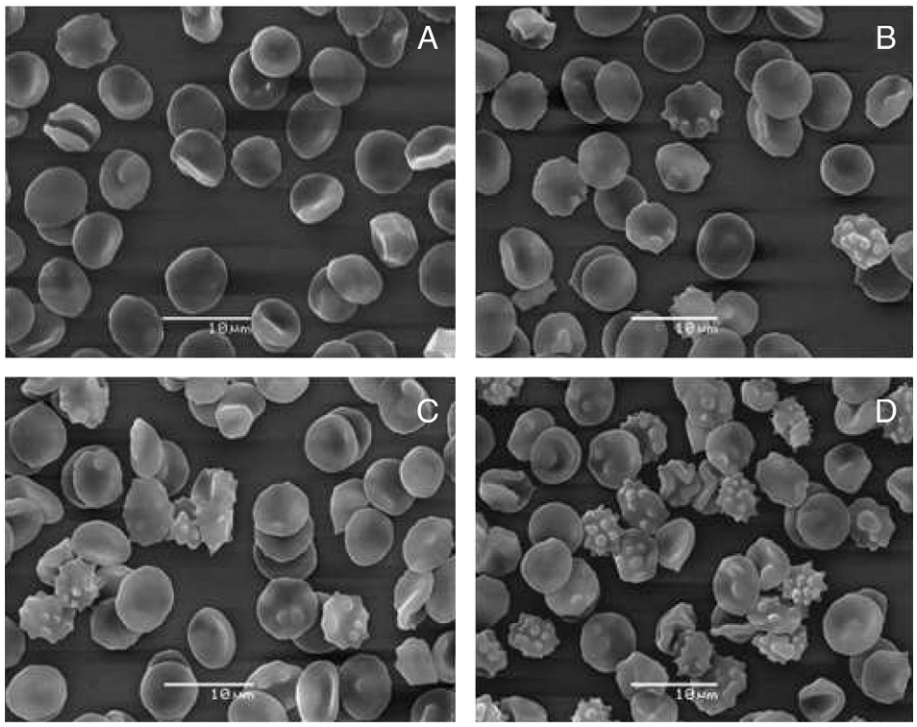

Fig. 4. Effects of resveratrol (RV) on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 0.5 mM RV, (C) 1 mM RV, and (D) 2 mM RV.

However, in the presence of 0.5 mM HClO and 0.5 mM RV the large majority of the cells are normocytes, echinocytes and knizocytes (shown by arrows in Figs. 5C and 5D). These results demonstrate the protective effect of RV against the shape perturbing effect of HClO upon human erythrocytes.

3.3. Optical and defocusing microscopy (DM) studies of human erythrocytes

As it can be appreciated in Fig. 6, 0.6 mM HClO changed the normal discoid shaped erythrocyte (Fig. 6A) into a stomatocyte (Fig. 6C). This

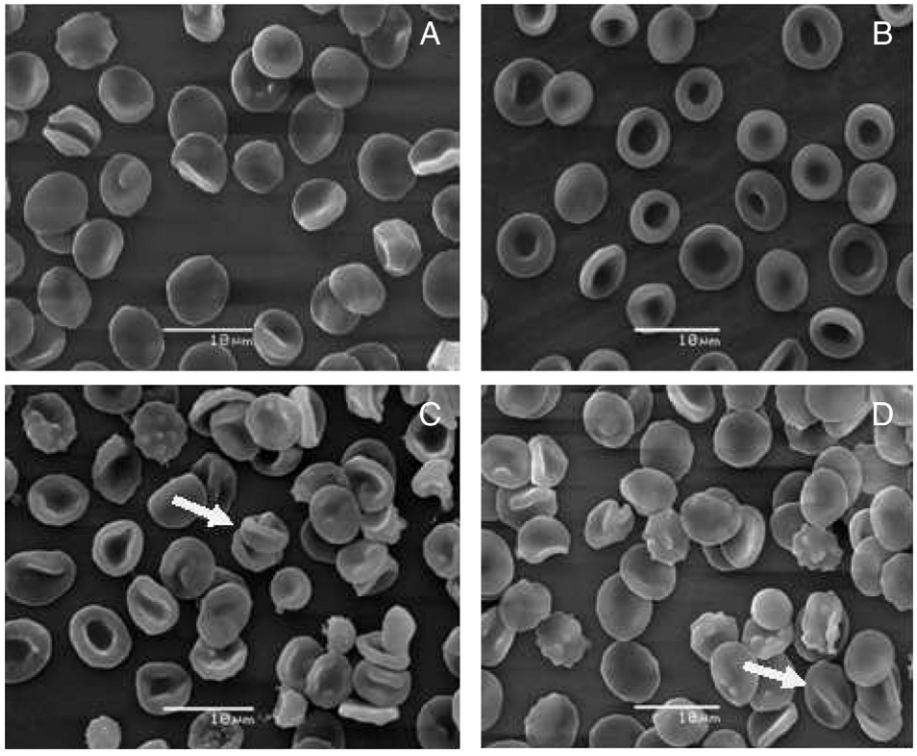

Fig. 5. Protective effects of resveratrol (RV) on the morphology of human erythrocytes. SEM images of (A) untreated erythrocytes; incubated with (B) 0.5 mM HClO; (C) 0.25 mM RV and 0.5 mM HClO; and (D) 0.5 mM RV and 0.5 mM HClO.

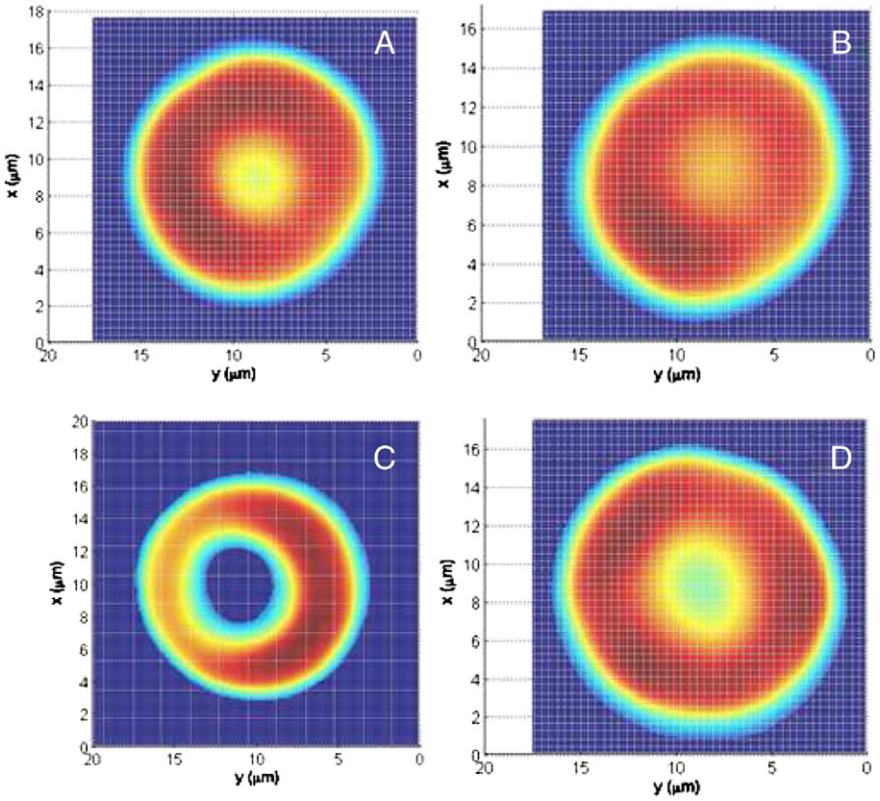

Fig. 6. Defocused images and three dimensional reconstructions of human erythrocytes; (A) untreated erythrocytes; (B) incubated with 20 μM resveratrol; (C) incubated with 0.6 mM HClO; and (D) incubated with 20 μM resveratrol and 0.6 mM HClO.

change was observed in the 3D reconstruction, where a flat part in the center of the cell can be appreciated. This is characteristic of a stomatocytic shape (blue color). However, if the RBC solution was incubated first with 20 μM RV, this molecule was able to inhibit the alteration of the erythrocyte shape (Fig. 6D). These results demonstrate the protective effect of RV against the shape perturbing effect of HClO upon human erythrocytes even in a very low concentration, which makes this molecule a very good antioxidant. It is important to note that 20 μM RV did not induce morphological changes to the cell (Fig. 6B).

3.4. Hemolysis assays

Increasing concentrations of RV in the 0.03 mM–2 mM range gradually increased the % of hemolysis although to a very low extent (about 8.5% with 2 mM RV, Fig. 7). However, the same range of RV concentrations gradually reduced the hemolytic effect of 2.5 mM HClO (100%), which reached a value of 12.5% with 1 mM RV.

4. Discussion

It has been reported that the main target of the RV action is the cell membrane [1,5,12]. Although most in vitro studies have been carried out in cancer cells [1,3,12], there are scanty reports on its effects on human red cells [12]. In the present study, the interaction and antioxidant property of RV was evaluated on human erythrocytes and molecular models of its membrane. The latter consisted in DMPC and DMPE bilayers, classes of lipids preferentially located in the outer and inner monolayers of the human erythrocyte membrane, respectively [17,18]. Results by X-ray diffraction on the interaction of RV with DMPC bilayers in the gel phase showed that RV produced a moderate but significant structural perturbation of the lipid bilayer, being higher this effect in the hydrophobic region of DMPC. On the other hand, no effects were observed in DMPE. DMPC and DMPE differ only in their terminal amino groups, these being $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their dry crystalline phases with the hydrocarbon chains mostly parallel and extended and the polar groups lying perpendicularly to them [34].

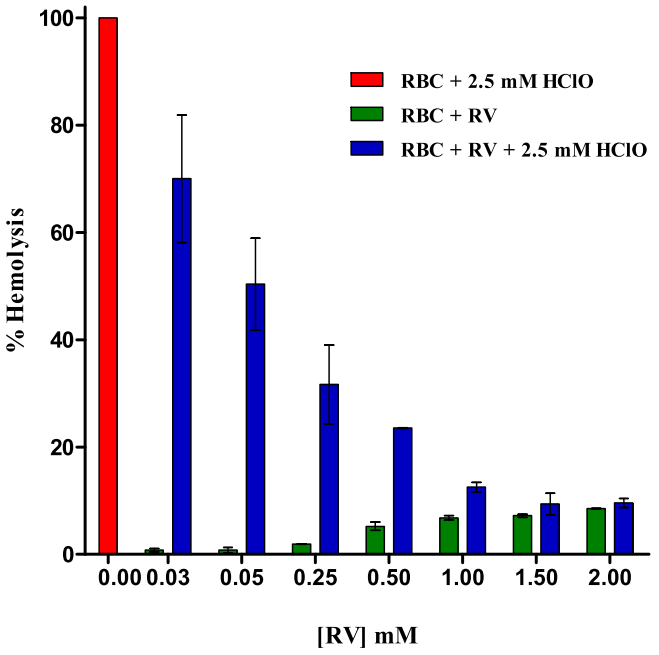

Fig. 7. Percentage of hemolysis of red blood cells (RBC) incubated with 2.5 mM HClO and different concentrations of resveratrol (RV).

However, DMPE molecules pack tighter than those of DMPC. This effect, due to DMPE smaller polar groups and higher effective charge, stands for a very stable multibilayer system which is not significantly affected by water [35]. The strong hydrogen network of DMPE bilayers is certainly a reason for the reduced penetration of water and other compounds into its interfacial headgroup region. On the other hand, the hydration of DMPC results in water filling the highly polar interbilayer spaces with the resulting width increase [35,36] (for further details regarding PC and PE fully hydrated gel phases, see Ref. [37,38]). This phenomenon might allow the incorporation of RV into DMPC bilayers and thus inducing the consequent structural perturbation. Similar results have been obtained by other techniques [5,12,39,40]. While some of these studies concluded that RV interacts with the polar headgroup region of PCs [5, 12,39], another report locates RV into the hydrophobic region of the bilayer [40]. Our own results showed that RV mostly inserted into DMPC hydrophobic acylic chains. The lipophilicity of RV, and therefore its potential for interaction with the hydrophobic part of DMPC can be evaluated by its corresponding partition coefficient. The logarithm of this value (log *P*) is used as a measure of hydrophobicity, and in the case of neutral molecules it is accepted as the value satisfactorily modeling their uptake into membranes. RV 1.87 log *P* in octanol–water and 2.63 in DMPC–water [40] points to its hydrophobic character and constitutes a proof that RV might preferentially intercalate into the hydrophobic matrix of the lipid bilayer. However, it is quite possible that one of the hydroxyl groups of RV, particularly the most acidic *p*-4 locates close to the negatively charged phosphate groups.

SEM observations showed that RV induced morphological alterations to red cells from their normal discoid shape to echinocytes. According to the bilayer couple hypothesis [41,42] the shape changes induced in erythrocytes by foreign molecules are due to differential expansion of the two monolayers of the red cell membrane. Thus, stomatocytes are formed when the compound inserts into the inner monolayer whereas spiculated-shaped echinocytes are produced when the compound locates into the outer moiety. The finding that RV induced the formation of echinocytes indicates that it was inserted in the outer leaflet of the erythrocyte membrane. This conclusion is supported by X-ray experiments carried out in DMPC and DMPE. In fact, results showed that RV only interacted with DMPC, which is preferentially located in the outer monolayer of the human erythrocyte membrane.

In the present study, the antioxidant capacity of RV was assayed on DMPC and DMPE bilayers and human erythrocytes exposed to HClO-induced oxidative stress. HClO is an extremely toxic biological oxidant generated by neutrophils and monocytes, and it is considered one of the most important factors causing tissue injuries in inflammation [43]. It is directly toxic to bacteria, endothelial cells, tumor cells and red cells. However, because it readily reacts with a range of biological targets it has been difficult to identify which reactions are critical for its cytotoxic effects [44]. Human erythrocytes are a reliable and easily obtainable model to detect oxidative stress [45]. Their simple internal structure depleted of nucleus and organelles provide an ideal system for this type of study. One major consequence of their exposure to HClO is lysis; although the exact mechanism is not clear, the cell membrane is considered the primary site for reaction. In fact, several studies have demonstrated that HClO treatment of erythrocyte membrane results in inhibition of Na^+, K^+-, and Mg^{2+}-ATPase activities, oxidation of SH^- groups, tryptophan residues, chloramine formation, changes of membrane fluidity and surface area, and membrane morphological transformations, and events that precede cell lysis [43,44,46]. As shown in Fig. 3A and C, HClO perturbs to different extents the structures of DMPC and DMPE bilayers, being this effect higher in DMPE. Since both lipids possess the same fully saturated acyl chains of 14 methylene groups, the explanation for the dissimilar HClO effect must be related to the structural differences in their headgroup regions. In the case of DMPE, the adjacent molecules and bilayers are attached by a network of electrostatic interactions and H-bonds between the amino (H-donating) and phosphate (H-accepting) groups resulting in a very stable flat gel phase. HClO polar molecules (pK 7.53) would disrupt the H-bond net that keep together DMPE molecules by being intercalated between the negatively charged phosphates and positively charged amino groups. On the other hand DMPC, which instead of hydrogens they are provided with bulky methyl groups, and with the presence of considerable amounts of water between the bilayers make the interbilayer interactions rather small. In this case, HClO molecules would remain mainly in the interbilayer water layers inducing perturbing effects at high concentrations by disruption of the intermolecular attractions in DMPC bilayer.

SEM and DM observations on human erythrocytes showed that HClO induced the formation of stomatocytes. This result is not surprising as the X-ray experiments demonstrated that HClO preferentially interacted with DMPE, class of lipid mostly located in the inner monolayer of the red cell membrane. According to the bilayer couple hypothesis [41,42], this location of HClO molecules should result in a lateral expansion of the membrane inner monolayer, changing the normal shape of erythrocytes into stomatocytes. SEM observations also demonstrated the protective effect of RV against the shape perturbing effect of HClO upon human erythrocytes (Fig. 5). Similar results were also obtained in DM observations (Fig. 6) and in hemolysis assay (Fig. 7). It might be then concluded that the location of RV molecules into the membrane bilayer might hinder the insertion of HClO and its consequent damaging effects. This conclusion can also imply that this restriction could apply to the diffusion of free radicals into cell membranes and the consequent decrease of the kinetics of free radical reactions. On the other hand, it has been proposed by Caruso et al. [7] that the antioxidant activity of RV is due to the electrophilic hydrogen atoms of its three hydroxyl groups. In their scheme, up to three hydrogen atoms per RV molecule can be transferred to reactive oxidants rich in electron density, being the *p*-4′ OH group the most acidic one. In our model, this group would be located in the surface polar region of the lipid bilayer, close to the negatively charged phosphate groups in the lipid–water interface, while the rest of the molecule would lie in the hydrophobic acylic chain region. In conclusion, RV would act by blocking access of the oxidants into the lipid bilayer and scavenges them before they can penetrate the cell membrane. It is worthwhile to mention that the assayed RV concentrations used in the human erythrocyte experiments fell within its human physiological level in plasma (10 μM–0.22 mM) [47].

Acknowledgments

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