In vitro action of 2.2'-azobis-2 amidinopropan dihydrochloride, red wine polyphenols, resveratrol and catechin on anion permeability for chloride in human red blood cell

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ABSTRACT

Introduction: The purpose of this study was to evaluate the ability of polyphenols (red wine polyphenols, resveratrol and catechin) on anion permeability for chloride and oxidative damage in human erythrocytes exposed to the water-soluble free radical initiator 2.2'-azobis-2 amidinopropan dihydrochloride (AAPH). **Materials and Methods:** The reducing agent treatment efficacy was observed by evaluation of anion permeability for chloride, lipid peroxidation and hemolysis in red blood cells (RBCs). Anion permeability for chloride is an indicator of membrane protein damage and is evaluated in RBCs by the specific absorption of methemoglobin (C_M) at 590 and 635 nm after treatment of heparinized blood with $NaNO_2$. The measurement of the membrane lipid degradation is obtained by the determination of malondialdehyde. The lipid peroxidation susceptibility is observed after the oxidative stress induced by AAPH. The hemolysis assays are conducted on blood samples in phosphate buffer saline and the morphological changes of erythrocytes are observed by optical microscopy as membrane damage verification parameter. **Results:** Polyphenol treatment is associated with a significant increase in anion permeability for chloride compared with control and AAPH affected cells. Treatment with polyphenols was associated with a significant reduction in mean ± standard error of the mean membrane lipid peroxidation compared with control and AAPH treatment. Hemolysis data are also obtained in the previously described conditions. RBCs morphology data indeed confirm previous observations. **Conclusion:** In this study, it is evident that after *in vitro* oxidative damage of the membrane, red wine polyphenol extracts are as effective reducing agents also on C_M indicator of membrane protein damages. Consequently, both red wine and polyphenol extracts both alone and mixed among them efficiently relieve the effects of oxidative stress.

Keywords: Anion permeability for chloride, hemolysis, lipid peroxidation and red blood cells, natural antioxidants, 2.2'-azobis-2 amidinopropan dihydrochloride oxidating agent

INTRODUCTION

The cell system exchanges Cl^- for HCO_3^- involving the cell membrane, while in the meantime the gas transport occurs in red blood cell (RBC) from circulating blood. Quantitative alterations of this protein are significant to preserve cell morphology.[1]

Authors have studied anion permeability for chloride in RBC under the action of reducing agents of red wine polyphenols during 2.2'-azobis-2 amidinopropan dihydrochloride (AAPH)-induced oxidation by the visible absorbance spectrum method evaluating the absorption of methemoglobin (C_M)[2] as an indicator of erythrocyte membrane anion permeability for chloride, and this is a unique work on RBC membrane permeability now available in the literature.

In literature[3] only one research on the protective effect from wine after AAPH-induced lipid and protein oxidation in low-density lipoprotein complexes (LDL) is present. At 40 μM resveratrol and flavonoids entirely reactivated the AAPH-oxidized LDL. Total fluorescence is attained with 20 mg/L of wine extract (WE) in the oxidation system. Results confirm that the resveratrol is more effective than flavonoids as an effective, reducing agent. Results moreover show that WE,

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which contains monomers and oligomers of flavonoids and phenolic acids that protect LDL by oxidation.

According to this potential activity, the aim of this work is to study the properties of main polyphenols (red wine polyphenols, resveratrol and catechin) as radical scavengers protecting RBC membrane from AAPH induced oxidative injury and preserving the RBC membrane anion permeability for chloride, lipid peroxidation products malonyldialdehyde (MDA), structural damages (hemolysis and RBC morphology).

MATERIALS AND METHODS

The research was carried out on 10 healthy subjects of both sexes between 35 and 60 years of age. All patients gave informed consent for participating in this study and for blood samples. The study was in accordance with the guidelines of the Institutional Ethical Committee of Europe. Venous blood was collected in heparinized tubes. Samples are centrifuged at 15°C for 10 min at 800 g to separate plasma, and buffy coat, and isolated RBCs were washed 4 to 5 times with 0.154 M NaCl.

Evaluation of RBC anion permeability for chloride

Anion permeability for chloride of RBC was determined according to the method described.[4] Shortly after, 0.05 mL of the heparin-treated blood and 0.05 mL of 0.06 mol/L $NaNO_2$ were added to 0.4 mL of phosphate buffer saline (PBS). The mixture was left to rest at 0°C for 5 min and after washed 3 times with PBS. Samples were lysed with 1% Triton X-100 for 2 h at 4°C. The methemoglobin (MetHb) specific absorption at 590 and 635 nm was determined in a Shimadzu ultraviolet-2100 spectrophotometer. The C_M was evaluated according to the equation:

$$C_M = \frac{E_X - 0.04E}{1.07E}$$

Where E_x is the absorption at 635 nm and E is the absorption at 590 nm. C_M is considered an indicator of RBC membrane anion permeability for chloride.[2]

Evaluation of lipid peroxidation

As previously described by our measurement of MDA, it is widely used as an indicator of lipid peroxidation.[5] MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts, including cross-linked products. The thiobarbituric acid reactive substances method is commonly used to measure MDA in biological samples.[6] The AL Detect (MDA specific) lipid peroxidation assay kit (AK-171)(Enzo Life Sciences, NY, USA) method is designed to assay free MDA, the assay conditions are useful to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals. The AK-171 assay is based on the reaction of a chromogenic reagent, N-methyl-2 phenylindole (NMPI) with MDA at 45°C. One molecule of MDA reacts with two molecules of NMPI to yield a stable carbocyanine dye. The AK-171 assay is specific for MDA because 4-hydroxyalkenals do not produce significant colour at 586 nm under the conditions of the assay.[7] Blood samples were obtained from healthy human volunteers by venipuncture into heparinized tubes and centrifuged at 2000 g for 10 min a 4°C. The plasma was removed, and packed erythrocytes were washed 3 times with (PBS; 10 mM sodium phosphate, 135 mM NaCl, pH 7.4). The buffy coat of white cells was removed. The washed erythrocytes were suspended in PBS at a final hematocrit of 5%,[8] that is the same experimental protocol used for determination of lipid peroxidation in RBCs with addition of the water-soluble exogenous starter AAPH (60 mM)(alternative Sigma-Aldrich®) a pro-oxidant useful to generate free radicals.[9]

Hemolysis test

For each measurement, 1×10^6 RBCs were collected and washed three times with PBS. In an Experimental protocol initially RBCs were pre-incubated at 37°C for 5 min in a water bath and successively two different series of micro test-tubes were performed: The first one treated with 20 volumes of distilled sterile H_2O and the second one treated with the same volume of an azo compound solution AAPH (60 mM) diluted in PBS at pH 7.4. Hemolysis assays were incubated up to different times (20 or 40 or 80 or 120 or 180 min). At the concentration of 60 mM, AAPH induces a hemolysis that occurs roughly after 2 h of exposure, and only for higher concentrations, hemolysis is observed earlier.[10] The extent of hemolysis was spectrophotometrically determined.[8] At specific intervals the reaction was taken out, diluted to 20 volumes with isotonic saline buffer and centrifuged (1000 g for 10 min). The percentage of hemolysis at different incubation intervals was determined by measuring the absorbance of the supernatant of the erythrocytes at 540 nm and compared with that of complete hemolysis, by lipid peroxidation, according to the literature.[8,9]

RBC morphology study

The state of RBC membranes was determined through a typical histological method defined May-Grunwald from Merck (Germany). This method is divided into several steps, in fact initially 5 µl of RBCs were put down on the slide, and the cover-slide had to stick to the blood drop with an angle of 40°, successively a blood crawl is carried out. The slide

is left for 5 min to dry; then the slide is fixed by addition of May-Grunwald die 3 times. Three 15 min staining cycles are done; each slide is washed with tap water at room temperature and dried. Finally, the cover-slide is soldered with Canadian balsam. RBCs morphology was captured by images advanced 3.2 on Motic AE21 reverted microscope and M580 camera, analysis by NIH ImageJ 1.43.[9]

Preparation of red wine

The wine used in this research is an 18-month-old (Savuto) red wine. The wine was made by traditional grape fermentation with stem contact. The Savuto is mainly produced from gaglioppo grape (*Vitis vinifera* L., cv) and is selected because it is one of the best varieties produced in Italy. The stem contact during grape skin in Fermentation provides many monomer and polymer proanthocyanidins[11-13] to the wine. The procedure of Fermentation and its post-treatment for bottling were carried out as described earlier.[13] The wine is aged in bottles for the period of 6 months prior to analysis.

Mean red wine polyphenol concentrations are about 2-7 mg/l for total resveratrol and about 10-250 mg/l for catechins.

Polyphenol concentration

The parameters were evaluated to describe the action of reducing polyphenolic compounds equivalent to reducing power of 5.2 mM Gallic acid from 20 µl of Savuto red wine.[14] The reducing action of polyphenols is equivalent to the physiological concentrations of trans-resveratrol (RESV) 5 µM and (+)-cathechin (CAT) 50 µM. The concentrations of reducing agents are chosen according to literature data. Reducing agents were provided from Sigma Chemical Co. (St. Louis, MO, USA) and AAPH was provided from Polysciences Inc. (Warrington, PA, USA) and used at the final concentration of 60 mM.[8]

Statistical analysis

Data are expressed as mean ± standard error of the mean. A one-way analysis of variance and Bonferroni *post-hoc* test for coupled data at each time was performed. At each time, ten observations were performed. Database managements and statistical analysis were performed using GraphPad Software Inc. "Prism 6" for Windows. Values were considered as statistically significant for $*P < 0.05$, $** P < 0.01$ and $*** P < 0.001$ levels.

RESULTS

To evaluate in human RBC the *in vitro* effects of AAPH and the antioxidant activity of polyphenols from red wine, authors start the research evaluating RBC anion permeability for chloride.

Figure 1 shows the values of membrane anion permeability for chloride in both groups (controls and AAPH-treated RBCs). The statistical analysis of data shows that AAPH *in vitro* treatment lowers the parameter in comparison to all controls. Furthermore, the reducing agent treatment (T2-T4) also significantly recovers the activity up to values higher than AAPH treatment. The recovery of anion permeability for chloride is always lower than control data.

Anion permeability for chloride values were slightly higher after red wine and resveratrol treatments (as reducing agents) in both experimental groups (controls and AAPH-treated RBCs), corresponding to a preserved architecture and anion permeability for chloride of the human erythrocyte membranes.

To confirm the efficacy of oxidant and reducing treatments on human RBC *in vitro* authors evaluated lipid peroxidation, hemolysis and RBC morphology.

In Figure 2, MDA levels are described in membranes from several varieties of treated and untreated RBC with AAPH, under the action of reducing agents or without treatment. On the contrary, as described in Figure 2, AAPH-induced increases of MDA levels are partially recovered by natural reducing agents at levels almost comparable to control the

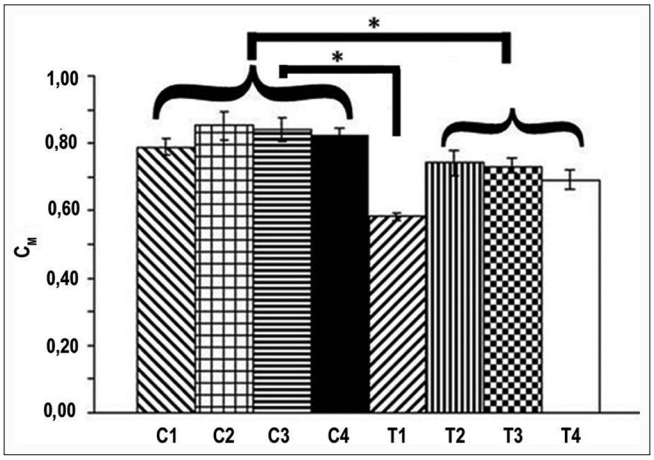

Figure 1: Red blood cell membrane anion permeability for chloride. Data are mean ± standard error of the mean on 10 determinations. *P < 0.05 according to one-way analysis of variance and Bonferroni *post-hoc* test coupled data at each time were performed. C = Control sample, T = Treated sample (Gallo *et al.*, 2013) C1 = Control; C2 = Control + Red Wine 5, 2 mM (reducing power equivalents of gallic acid); C3 = Control + Resveratrol 5 µM; C4 = Control + Catechin 50 µM; T1 = AAPH 60 mM; T2 = AAPH 60 mM + Red Wine 5,2 mM (reducing power equivalents of gallic acid); T3 = AAPH 60 mM + Resveratrol 5 µM; T4 = AAPH 60 mM+ Catechin 50 µM

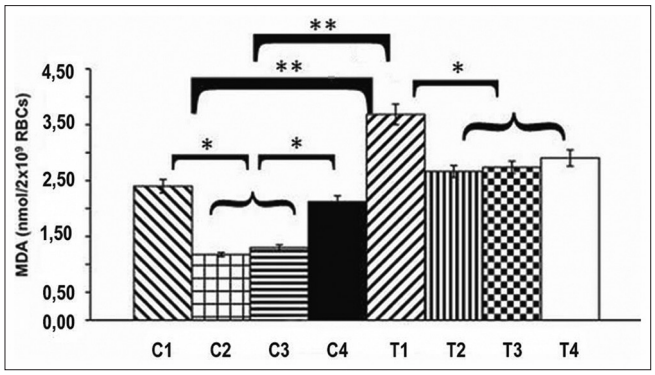

Figure 2: Effects of lipid peroxidation and induced by 2.2'-azobis-2 amidinopropan dihydrochloride (60 mM) (t = 60 min) on the malonyldialdehyde levels of human red blood cell membranes. Data are mean ± standard error of the mean on 10 determinations. Column labels are according to $^{*}P < 0.05$ and $^{**}P < 0.01$ according to one-way analysis of variance and Bonferroni *post-hoc* test for coupled data at each time were performed (Figure 1)

group. C2-C4 results always slow down MDA levels in RBC membranes, but CAT is less effective. Red wine, among them, is almost as powerful as resveratrol alone in both groups (controls and AAPH-treated RBCs).

As shown in Figure 3 time courses of RBC hemolyses are described at several incubation times (60, 120, 180 and 240 min) either under the action of natural oxidative pathways or by treatment with radical donor AAPH (60 mM). This experiment shows also the natural reducing agents partially recover the effect of both oxidative and reducing agent treatments. As described in all experiments, AAPH-induced hemolysis is more relevant than in other treated samples. As described in previous experiments (Figures 1 and 2) resveratrol and red wine polyphenol treatments are more efficient than CAT in preserving RBC membrane structural recovery at 60 and 120 min of incubation.

Figure 4 shows the effect on control RBC (A) and AAPH 60 mM treated RBC (B) on structural membrane continuity in 60 min incubation at 37°C, according to surface plots of RBC densitograms evaluated by NIH Image J 1.43 program. The image evaluation was performed on phase contrast microscopical photographs acquired on Motic AE21 reverted microscope and M580 camera. In these evaluations, AAPH lysis damages are more relevant than control ones.

DISCUSSION

In the present study, polyphenol (resveratrol and red wine) treatment is associated with an improvement in erythrocyte anion permeability for chloride-related with cell membrane derangements.

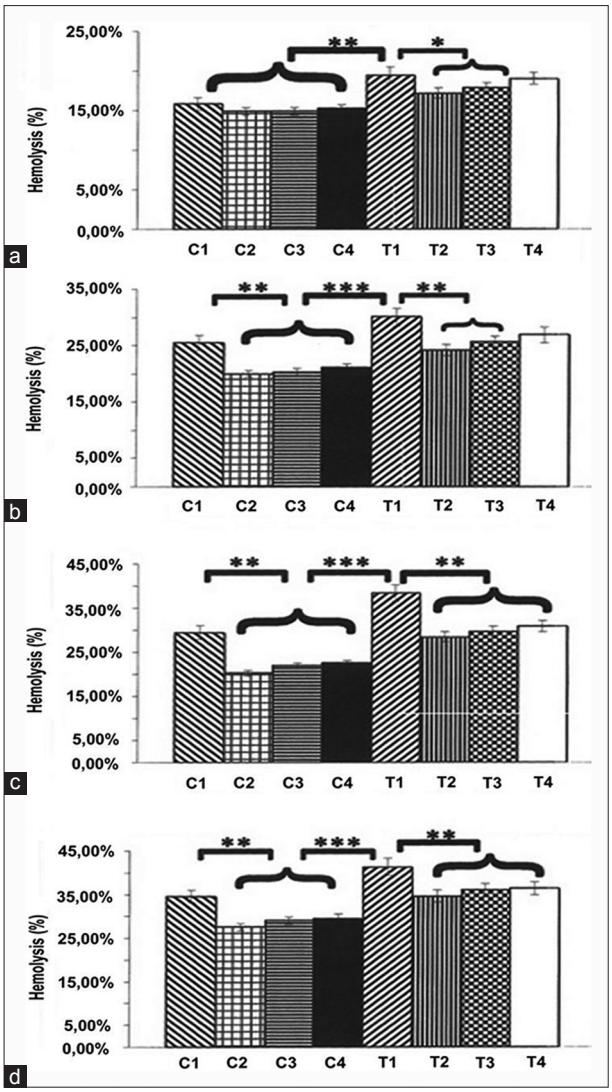

Figure 3: Effect of 2.2'-azobis-2 amidinopropan dihydrochloride-induced hemolysis in sportive human red blood cells at several times. Data are mean ± standard error of the mean on 10 determinations. Column labels are according to incubation times a = (60 min); b = (120 min); c = (180 min); d = (240 min). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0{,}001$ according to according to one-way analysis of variance and Bonferroni *post-hoc* test for coupled data at each time were performed (Figure 1)

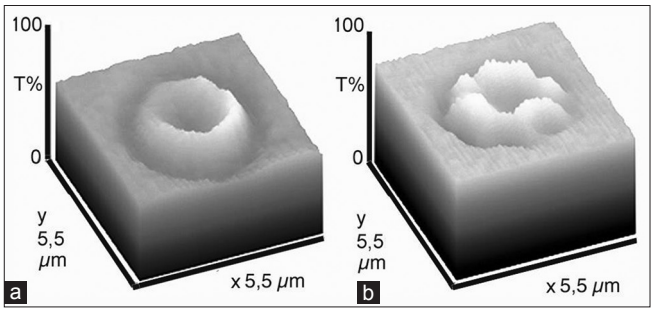

Figure 4: Red blood cells surface plots of 60 min incubation at 37°C in control (a) and in presence of 60 mM 2.2'-azobis-2 amidinopropan dihydrochloride (b)

The cell system exchange of Cl^- for HCO_3^- represents a target of membrane oxidative damage and is evaluated in RBCs by the specific C_M at 590 and 635 nm after treatment of heparinized blood with $NaNO_2$.

According to our research, the *in vitro* action model of artificial agent AAPH on RBC plasma membranes shows that anion permeability for chloride (evaluated by C_M^2) decreases after treatment and is partially recovered after administration of either resveratrol or red wine polyphenols extracts.

Our data confirm those on LDL peroxidation[3] with AAPH by MDA, hemolysis and RBC morphology study from our laboratory.[9,15]

Moreover in the present work all previous results are compared, so that a relationship among them is evident and correlate each other, so that the action of red wine polyphenol extracts are demonstrated as effective reducing agents both alone and in mixture.

MDA, hemolysis and RBC morphological study confirmed the resveratrol and red wine efficacy on improvement of RBC membrane integrity against *in vitro* oxidative stress damages.

The described damages could be linked to the erythrocyte membrane framework (Figure 5), so a lack of adequate lipid organization can significantly influence ion permeability. The alteration of membrane fluidity consequent to lipid oxidation represented by MDA level increase can reflect the change of the structure and function of membrane lipids and proteins. The permeability is another important property of erythrocyte membrane. Depending on the exchanger ($Cl^- \rightarrow\leftarrow HCO_3^-$), that transports anions across the membrane, it is related to the framework of erythocyte membrane. To sum up, the alteration of anion permeability can reflect the change of erythrocyte membrane protein framework. Moreover, our results could describe the overall effect of the three described alterations together.

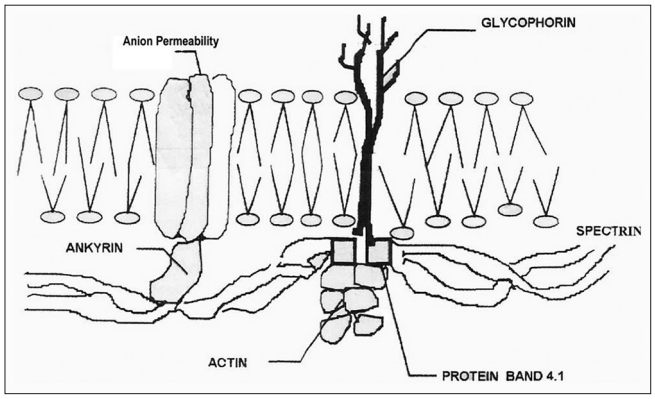

Figure 5: Erythrocyte membrane organization

CONCLUSION

According to described results, red wine polyphenols act on CM indicator of anion permeability as effective reducing agents. Red wine polyphenol extracts effeciently relieve the effects of oxidative stress.

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