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Antioxidant effect of red wine anthocyanins in normal and catalase-inactive human erythrocytes

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Abstract

Previous studies reported that aged red wine, but not novel red wine or white wine protects human red blood cells from oxidative damage induced *in vitro* by H_2O_2. Here, we demonstrate that the beneficial properties of aged red wine are due, at least in part, to the presence of anthocyanins. We firstly measured the "antioxidant power" of an Italian red wine (Taurasi, Avellino) and that of its anthocyanin fractions by using Ferric Reducing Antioxidant Power Assay. Subsequently, we demonstrate that fractions containing anthocyanins lower ROS (reactive oxygen species) and methemoglobin production in human erythrocytes treated with H_2O_2. Finally, we reported that the protective effects of anthocyanins were also confirmed in an experimental model in which RBCs were deprived of catalase activity by treatment with 4 mM sodium azide. The results obtained clearly demonstrate that red wine anthocyanins protect human RBCs from oxidative stress.

Keywords: Red wine anthocyanins; Antioxidants; Erythrocyte; Reactive oxygen species (ROS); Catalase

1. Introduction

The aerobic metabolism of living organisms constantly produces reactive oxygen species (ROS), such as hydrogen peroxide, organic peroxide, superoxide anion and hydroxyl radical which are receiving particularly attention because of their possible involvement in several diseases including certain hemolytic anemias [1–3]. Protection against ROS is provided by an array of different compounds contained in the human diet [2–5], and by enzymatic antioxidants, such as catalase and glutathione peroxidase. Several reports indicate that catalase is the predominant enzyme that catabolizes exogenous H_2O_2 in eukaryotic cells including RBCs (red blood cells) [6–9].

Human diet is rich of a great variety of micronutrients with antioxidant properties [10]. Among these, flavonoids hold an important role. They represent a large group of polyphenolic antioxidant compounds widely distributed in fruits, vegetables and beverage such as tea, beer and wine [11]. Several studies indicate that, due to its antioxidant properties, red wine is able to inhibit LDL oxidation [12,13], block the genotoxic effects of environmental mutagens [14], and reduce the incidence of cardiovascular diseases [15,16].

In a previous work, we demonstrated that aged red wine protects RBCs against the oxidative damage induced by H_2O_2 compared to white wine and novel red wine. Since aged red wine contains high levels of polyphenols, we hypothesized that its protective effects was associated to the presence of these compounds [17]. In the present work, we better define the role a specific class of polyphenols, namely anthocyanins, in protecting RBCs against ROS damage.

Anthocyanins are among the most abundant components of red wine [18], and are responsible for different grape colors [19]. Anthocyanins are flavonoids present in two chemical forms: glycoside and acylglycoside of anthocyanidins [20]. Some anthocyanidins (aglycone), with different hydroxyl or methoxyl groups, are reported in Fig. 1. The most abundant sugars present on the anthocyanins are glucose, rhamnose, xylose, galactose, arabinose and fructose. The sugar moiety is esterified on residues 3, 5, 7, 3′ and 5′. The glycosides are more stable than the corresponding aglycone [21]; in fact, glycosilation and acylation are two ways to enhance the solubility of anthocyanins in the wine [22]. The most abundant anthocyanins present in a typical Italian red wine are malvidin-3-O-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-glucoside and delphinidin-3-O-glucoside [18].

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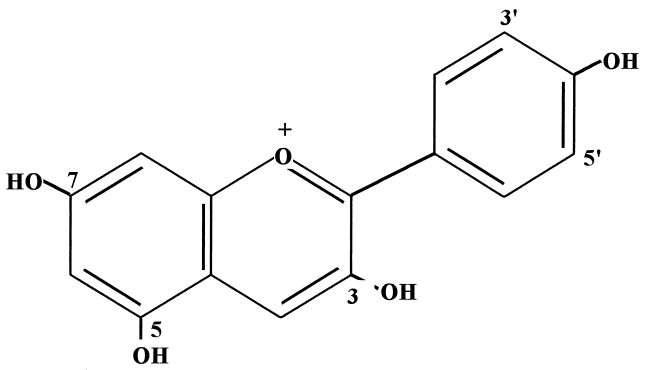

Aglycone	3'	5'
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H

Fig. 1. Structure of few common anthocyanidins with their substitution patterns.

In this paper, we reported that anthocyanins are responsible for the antioxidant properties of red wine in normal erythrocytes, as well as in those deprived of catalase activity.

2. Materials and methods

2.1. Chemicals

Dichlorofluorescein-diacetate (DCFDA) was purchased from Molecular Probes (Eugene, OR, USA); H_2O_2 and quercetin from Sigma Chemical Co. (St Louis, MO, USA); sodium azide from Applichem GmbH (Darmstadt, Germany); malvidin-3-O-glucoside, peonidine 3-O-glucoside, delphinidin 3-O-glucoside were a gift of Dr. D. Rotilio (Istituto di Ricerche Farmacologiche Mario Negri, Chieti, Italy). All other chemicals used were of research highest purity grade.

2.2. Preparation of erythrocytes

Human blood was drawn by venipuncture and the erythrocytes, after centrifugation, were washed in PBS (phosphate buffer saline) to remove plasma, platelets and buffy coat. Aliquots of blood containing 1×10^5 cells/μl were used in all experiments presented.

Table 1
Retention times of three standards of anthocyanins and their presence in total wine (TRW) and its purified fractions (A, B, IV)

	Retention Time (min)				
	Standard	TRW	Fraction A	Fraction B	Fraction IV
Delphinidin 3-O-glucoside	20.00	19.62	20.03	—	—
Peonidin 3-O-glucoside	27.20	27.18	27.30	—	—
Malvidin 3-O-glucoside	27.90	27.69	28.07	—	—

2.3. Preparation of wine samples and wine fractions containing anthocyanins

We used in all experiments aliquots of a Taurasi red wine (TRW, Feudi San Gregorio, Sorbo Serpico, Vintage 1994, Avellino, Italy), lyophilized to eliminate ethanol, and resuspended in the same volume of 0.01 N HCl (pH 2.0). The fraction of red wine rich in anthocyanins was prepared as described and named fraction IV according to the original definition [23]. Briefly, 0.5 ml of TRW was applied to Sep-Pak C-18 (Waters Corporation, Milford, MA, U.S.A.), and fraction IV was eluted with 5 ml of 100% methanol, lyophilized and resuspended in 0.5 ml of PBS. Total anthocyanin red wine, and non-anthocyanin red wine were prepared according to Ghiselli et al. [18] Briefly, 2 ml of TRW were extracted at least three times with ethyl acetate; the organic phases, containing non-anthocyanin compounds, were pooled together, dried and resuspended in 2 ml of PBS (fraction B). The aqueous solution, containing total anthocyanins was lyophilized and resuspended in 2 ml of HCl, pH 2.0 (fraction A). To measure anthocyanins present in the isolated fractions, we measured absorbance of TRW, fraction IV and fractions A and B at 520 nm, as reported [24]. Aliquots of different fractions were stored at -20°C.

2.4. HPLC analysis of TRW and its fractions

In order to verify the presence of anthocyanins in the fractions isolated from red wine fractions, we employed an HPLC method (Table 1). TRW and fractions IV, A and B were analyzed by reverse phase HPLC using a Gold System 126 equipped with a UV detector 166 (Beckman, Palo Alto, CA, USA). Each sample was applied onto a Kromasil C_{18} column (4.6 mm $\times$ 25 cm). Detection was carried out at 280 nm [49]. A gradient solvent system for the separation of compounds (solvent A: H_2O + TFA 0.1%; solvent B: 95% acetonitrile + 0.1% TFA) was employed. The elution profile, performed at 0.8 ml/min flow rate, was as follows: 0–7 min 10% B; 7–32 min, 10–25% B; 32–37 min, 25–100% B; 37–42 min 100% B; 42–47 min, 100–10% B. All solvents were of HPLC grade, filtered through a 0.45 mm filter (Millipore, Molsheim, France) before use. Malvidin-3-O-glucoside, peonidine 3-O-glucoside, delphinidin 3-O-glu-

coside were used as standards and their retention times are reported in Table 1.

2.5. Determination of hemolysis and Met-Hb contents

The extent of hemolysis was evaluated as previously report [17]. Briefly, RBCs ($1 \times 10^5/\mu l$) resuspended in 0.4 ml of PBS, were incubated with 10 μl of TRW, or 10 μl of red wine fractions in presence or absence of 4 mM sodium azide for 20 min at 37°. After incubation, RBCs were centrifuged, washed and treated with different concentrations of H_2O_2 for different times, at room temperature. One duplicate was centrifuged and hemolysed using ice cold H_2O. Hemolysates were microcentrifuged at 13,000 $\times$ g for 2 min; the others, were centrifuged at 2,000 $\times$ g for 2 min and gently resuspended in PBS. Hemolysis was determined by spectrophotometric measurement at 540 nm, and by Coulter Counter (Coulter Onix, Miami, FL, USA). The met-Hb content was determined as a percentage of total hemoglobin (Hb), from the ratio of Hb released from the erythrocytes to the total Hb in samples incubated at various times and concentrations of H_2O_2, according to Winterbourn [25].

2.6. Fluorescent measurement of intracellular ROS

We employed DCFDA to measure ROS production [26]. RBCs, resuspended in 0.4 ml of PBS, were incubated with 10 μl of TRW, or anthocyanin fractions plus 4 mM sodium azide for 10 min a 37°C. After incubation with 10 μM DCFDA, RBCs were centrifuged, resuspended in 2 ml of PBS, and treated with 10 μM H_2O_2 for 5 min at room temperature. ROS production was measured fluorometrically with excitation and emission settings at 495 and 530 nm, respectively (Perkin Elmer LS 50B fluorimeter, Norwalk, CT, USA) and expressed as arbitrary units [17].

2.7. FRAP assay

In vitro "antioxidant power" of TRW and its anthocyanin fractions was determined by FRAP assay (Ferric Reducing Antioxidant Power) accordingly to Benzie et al. [27] Briefly, 10 μl of TRW, or TRW fractions, were added to a total volume of 1 ml. The "antioxidant power" was calculated as difference in absorbance at 593 nm between the reading at 6 min and the reading at 0 min. These values were related to a standard curve made with a pure solution of quercetin.

Table 3
FRAP assay on TRW and anthocyanin fractions measured as equivalent of standard quercetin

	*FRAP values
Taurasi Red Wine (TRW)	48.0
Fraction A	30.0
Fraction B	9.7
Fraction IV	26.0

* Values are expressed as concentration (μM) of quercetin equivalent.

2.8. Catalase activity

Catalase activity was expressed as the first-order kinetic constant of the rate of disappearance of 10 mM H_2O_2, as measured by absorbance at 240 nm [28]. After incubation with TRW and its fractions (10 μl), RBCs were solubilized in 1 ml of ice cold H_2O. Hemolysed samples were centrifuged a 13,000 $\times$ g for 2 min, and supernatants were diluted 1:10 in PBS. Changes in absorbance were measured after 20 s from addition of H_2O_2 10 mM.

3. Results

In this study, we used an aged red wine (TRW) whose polyphenol content was fractionated as described in the Method section. To characterize the antioxidant activity of anthocyanins and compare it to other wine components, we employed two different separation methods [18,23], that allowed us to obtain a fraction containing total anthocyanins (monomers and polymers) [15], one deprived of anthocyanins, and another enriched in polymeric anthocyanins, produced by reaction between monomeric anthocyanins and flavonoid compounds [39–41]. Table 2 summarizes the properties of TRW and fractions IV, A and B in terms of type and concentration (spectrophotometric measurement at 520 nm) of anthocyanins. As indicated, fraction B contained an amount of anthocyanins 10 times lower respect to the other samples that showed comparable concentrations of anthocyanins. In addition, as reported in Table 2, we dem-

Table 2
Anthocyanin composition and concentration in TRW and its purified fractions. Their potential toxicity on RBCs is reported as % of viability.

		Anthocyanin Concentration (μM)	RBC Viability (%)
Taurasi Red Wine (TRW)	All wine components	3.93	100
Fraction A	Monomers of anthocyanins and other components	3.93	96
Fraction B	Absence of anthocyanins	0.34	100
Fraction IV	Polymers of anthocyanins	3.55	100

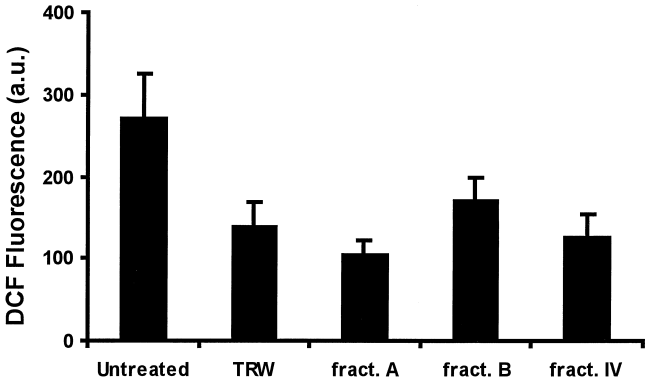

Fig. 2. Reactive oxygen species measurement after incubation of RBCs with TRW and anthocyanin fractions, in the presence of 10 μM H_2O_2 for 5 min. Determination was as described in Methods. Values are means + SEM (n = 3).

onstrated that neither TRW, nor its fractions showed any toxic effect towards RBCs.

3.1. Antioxidant effects of TRW and its anthocyanin fractions

Previous works reported that FRAP assay is a valid method to determine the antioxidant properties of tea [29]. Therefore, we used the same method to measure the antioxidant power of TRW and its fractions. As reported in Table 3, whole TRW extract, fraction A and IV showed a high antioxidant power compared to fraction B, accordingly with the concentrations of anthocyanins present in the different samples (Table 2).

RBCs are susceptible to oxidative damage as a result of the high concentrations of hemoglobin, a promoter of oxidative processes [30]. This raises the possibility that antioxidants might prevent oxidative damage in RBCs**.** Therefore, we evaluated the antioxidant effect of TRW, fractions A, B and IV after addition of H_2O_2, at a concentration comparable to that possibly present *in vivo* (10 μM) [31]. As reported in Fig. 2, intracellular ROS were mainly scavenged by TRW, fraction A and IV rather than fraction B. Fraction A, containing all the anthocyanin compounds, showed the most relevant antioxidant activity compared to fraction IV and TRW. To further confirm the antioxidant properties of anthocyanins, we measured the methemoglobin production in RBCs after treatment with H_2O_2. TRW and its fractions did cause neither hemolysis, nor variations of methemoglobin concentrations in the absence of H_2O_2 (data not reported). On the opposite, after incubation for 3 hr at room temperature in the presence of 100 μM H_2O_2, a remarkable increase of methemoglobin contents was detected. Incubation with TRW, fraction A and fraction IV abolished methemoglobin increase, rather than fraction B, according with the minor antioxidant properties of this fraction (Figure 3).

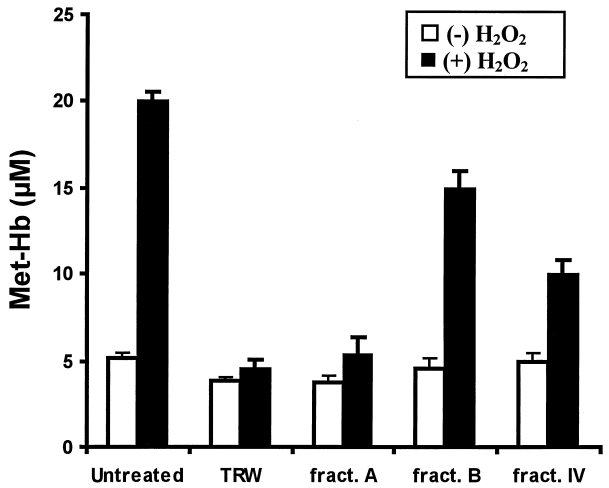

Fig. 3. Methemoglobin concentration of human RBCs in presence or in absence of H_2O_2 100 μM for 3 hr. Cells were incubated with TRW and anthocyanin fractions, as described in Methods. Values are means ± SEM (n = 3).

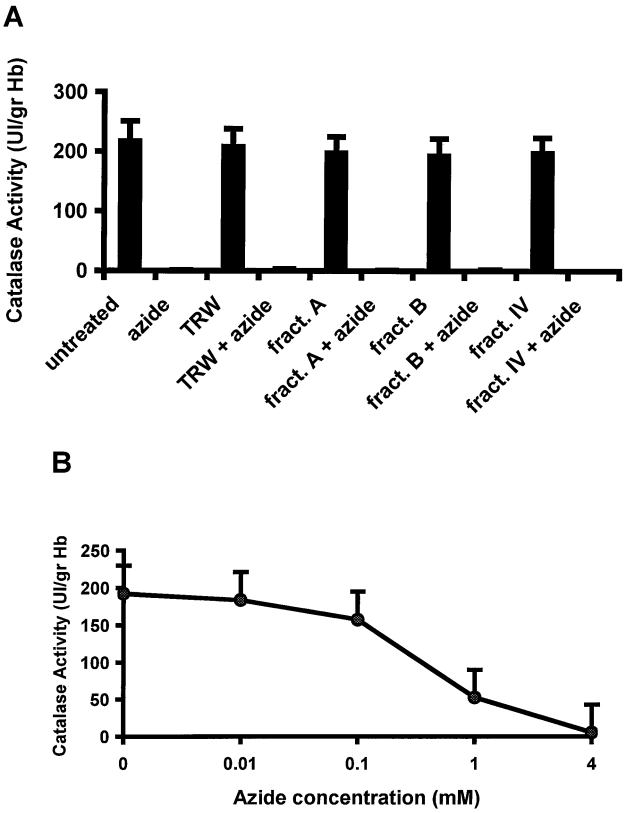

Fig. 4. Effect of TRW and anthocyanin fractions on RBC catalase activity. In panel **A,** RBCs were incubated in the presence of 4 mM sodium azide, plus and minus TRW and wine fractions for 20 min at 37°C. Catalase activity was determined as described in Methods section. Panel **B** shows a dose-effect curve of sodium azide on RBC catalase activity. Values are means ± SEM (n = 4).

3.2. Effect of TRW and anthocyanin fractions on catalase-deprived RBCs

Lack of erythrocytes antioxidant capacity and catalase inactivity have been associated to severe oral gangrene

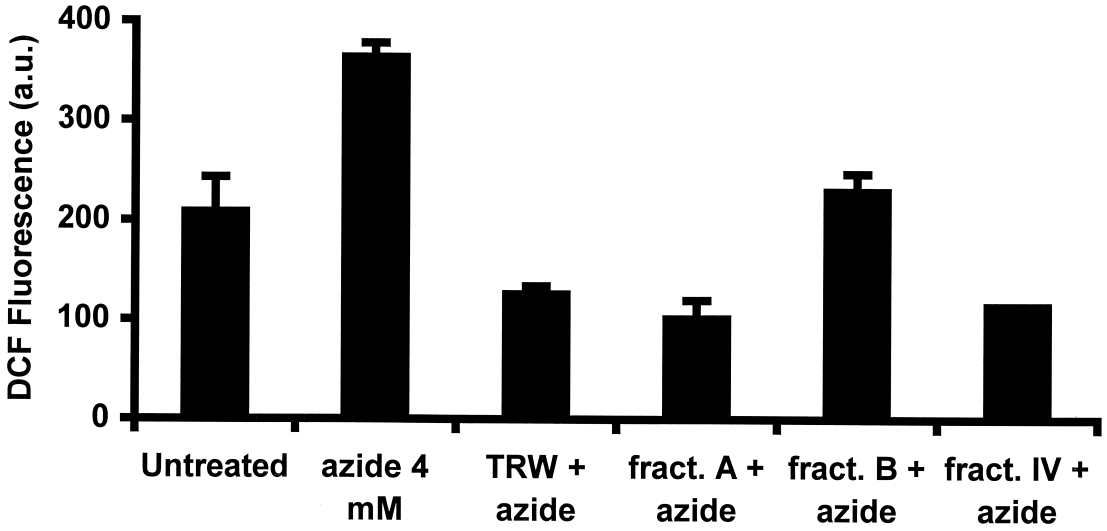

Fig. 5. Reactive oxygen species measurement after incubation of RBCs treated with TRW and anthocyanin fractions, in the presence of azide 4 mM after addition of 10 μM H_2O_2 for 5 min. Determination was as described in Methods. Values are means ± SEM (n = 3).

[32,33]. Based on these observations, we evaluated the antioxidant properties of TRW and its fractions on an erythrocytary model where catalase activity was inhibited by sodium azide, a specific inhibitor of this enzyme [34]. Firstly, we showed that TRW and its fractions did not interfere neither with catalase activity, nor with the activity of its inhibitor (Figure 4A), that, in our conditions, was employed at a concentration of 4 mM, a value able to totally inhibit catalase activity (Figure 4B). This inhibition was maintained, without toxic effects on RBCs for at least three hours (data not shown).

To demonstrate that the potential protective effects of anthocyanins were also verified in a catalase-inactive model, we measured ROS release in RBCs after treatment with H_2O_2 in the presence of sodium azide. As reported in Figure 5, the addition of sodium azide significantly increased the level of ROS, as expected. However, the incubation with TRW and fractions A and IV was able to reduce ROS levels to control values. On the opposite, fraction B, less rich in anthocyanins, failed to show the same protective effect (Figure 5). To further confirm these observations, we measured the production of methemoglobin in the presence of sodium azide (Figure 6). The damage caused by H_2O_2 addition, and enhanced by sodium azide treatment, was almost totally abolished by incubation with TRW and anthocyanin rich fractions (A and IV). Fraction B was unaffected according to its minor antioxidant property (Figure 6).

4. Discussion

Among food rich in polyphenols, red wine contains flavonoids and non flavonoids molecules, including anthocyanins that are present at a concentration of 100–1,000 mg/lt [35]. Anthocyanins are natural polyphenolic compounds that have been shown to have healthy effects as, antiinflammatory [36], and antidiabetic agents [37], with a daily intake in human evaluated to be as much as 180–215 mg/day in US [38]. In this study, we demonstrated that fractions containing anthocyanins derived from an Italian aged red wine, show strong antioxidant properties in RBCs treated with micromolar doses of H_2O_2. This effect has been also proved in erythrocytes deprived of catalase activity, an enzyme essential in removing H_2O_2-generated oxidant species [7]. The incubation of RBCs with a non toxic concentration of sodium azide abolished

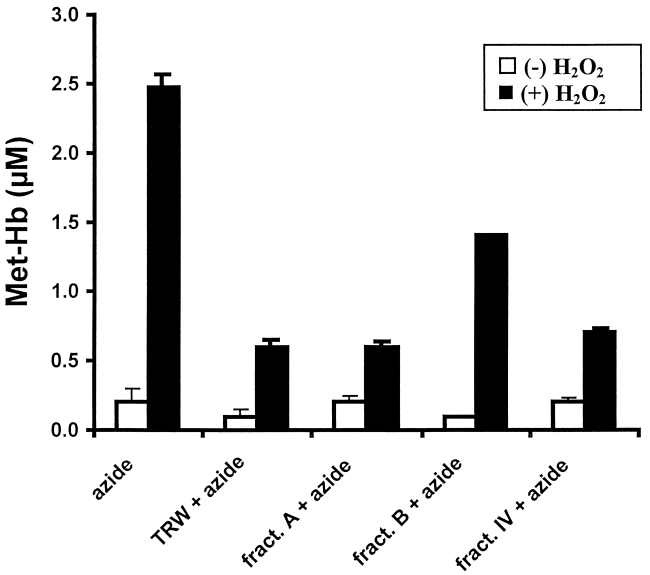

Fig. 6. Methemoglobin concentration of human RBCs in presence of sodium azide 4 mM after addition of H_2O_2 10 μM for 3 hr. Cells were incubated with TRW and anthocyanin fractions, as described in Methods. Values are means ± SEM (n = 3).

totally catalase activity, representing a good experimental model in acatalasemic patients of Japanese variety (catalase activity ranging between 0.3–2.0% of normal values), or Swiss variety (catalase activity ranging between 0.5–2.0% of normal values) [7].

Using a liquid-liquid extraction protocol, we obtained two separate fractions: fraction A containing total anthocyanins, and fraction B representing the remaining red wine component deprived of anthocyanins [20]. The absence of anthocyanins in fraction B was demonstrated by HPLC analysis using typical standards (Table 2). We reported that fraction A, rich in anthocyanins, presented strongest antioxidant properties than fraction B, and nearly comparable to that of total red wine. These data are in agreement with a previous study showing that red wine anthocyanins have higher antioxidant properties than all other wine components when tested for hydroxyl and peroxyl scavenging, inhibition of LDL oxidation, and platelet anti-aggregation [20].

Once anthocyanins leave the surroundings of the grape cells in which they are stabilized, they undergo a series of reactions. Thus, during the making of red wine, the monomeric anthocyanins are transformed into polymeric anthocyanins by reaction with other flavonoid compounds and aldehydes during wine aging [39–41]. We found that fraction IV, rich in anthocyanin polymers [24], also presented a remarkable antioxidant activity, comparable to TRW. A fraction, named II and prepared following the same method [24], showed significant antioxidant properties; however, we did not include the effects of fraction II in this work since this fraction contained monomeric anthocyanins as well as other flavonoids, *e.g.* catechins and procyanidins, with similar antioxidant activity. Two other fractions, I and III, containing phenolic acids and flavonols, respectively, were toxic for RBCs in the presence of sodium azide 4 mM (data not shown).

In human plasma, appreciable levels of catechin [42,43] and caffeic acid [44] have been detected after ingestion of red wine. On the opposite, only few reports demonstrated the presence of anthocyanins in human plasma. Paganga et al. [45] found unidentified anthocyanins in plasma from normal individuals on non-supplemented diet, and Cao et al. [46] found anthocyanins in plasma after oral administration of an elderberry extract. More recently, Lapidot detected red wine anthocyanins in human urine [47]. Since these molecules were found after 1–3 hr from ingestion of red wine, we can conclude that, in our experiments, twenty minutes of incubation with TRW, or its fractions, represents a good approximation respect to the bioavailability observed *in vivo*.

The amount of TRW, or anthocyanin fractions used in our assays (10 μl) has been selected in order to bring the concentration of malvidin-3-O-glucoside, the most abundant anthocyanin species present in a typical aged red wine, to a value of 2 mg/L. The anthocyanin concentration in human blood, after ingestion of a regular amount of red wine (1–2 glasses), is 2–3 mg/L, a value very close to that reported in our experimental conditions.

In a previous report, we demonstrated that other components different than resveratrol and quercetin might account for the protective effect of red wine against oxidative damage [18]. Here, we show that anthocyanins are a good candidate to explain the antioxidant properties of TRW on RBCs challenged with H_2O_2. In fact, accordingly to our previous report, the minor protective effect of white wines respect to red wine, is probably due to the lower concentrations of total anthocyanins [24,48].

Acknowledgments

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