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Original Research

Concentration and time dependent cytoprotective effects of anthocyanins against oxidative hemolysis induced by water and lipid soluble free radical initiators: an *in vitro* study

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Abstract
The non-enzymatic, free radical-mediated peroxidation of erythrocyte membranes is now correlated with a variety of pathological conditions, and considered as a primary event associated with hematotoxicity of variety of drugs and chemicals that induce erythrocyte hemolysis. The plant derived polyphenols including anthocyanins have been suggested to enhance red blood cell resistance to oxidative stress both *in vitro* and *in vivo*. The present study was designed to investigate the concentration and time dependent anti-hemolytic activity of anthocyanins using *in vitro* models of azo compounds-induced erythrocyte membrane peroxidative damage. Three different concentrations (1, 0.1 and 0.01 mg/ml) of anthocyanins solution were incubated with erythrocytes suspension 30 min before addition of either water soluble (AAPH) or lipid soluble (AMVN) free radical initiators. The level of hemoglobin released into the solution was determined spectrophotometrically, both in presence and absence of anthocyanins. Moreover, the most effective concentration was selected to be added at different time intervals (zero time, 2 and 3 hours) post challenge with either AAPH or AMVN and the extent of hemolysis was determined as mentioned before. The results showed that anthocyanins, concentration dependently attenuated hemolysis induced by azo compounds, and substantial cytoprotective effects of anthocyanins were observed when they added simultaneously with azo compounds, while delayed addition of anthocyanins for 2 and 3 h diminished but not abolished the protective effect of anthocyanins. In conclusion, anthocyanins can effectively display concentration and time dependent cytoprotective properties against azo compounds-induced peroxidative hemolytic damage.

INTRODUCTION

Increasing evidence suggests that free radical mediated oxidative damage of membrane lipids and proteins eventually contributes to the initiation and propagation of numerous degenerative disorders, including cardio-vascular disease, cancer and aging [1, 2]. Erythrocytes are often used as biological model to investigate oxidative damage in biomembranes because of their high vulnerability to peroxidation, since their membranes are rich in polyunsaturated fatty acids (PUFA), and they have high concentrations of oxygen and hemoglobin (Hb) which promote the oxidative process [3]. The oxidation of erythrocyte and its ghost membranes induced by free radicals have been studied; and it has been found that reactive oxygen species (ROS) generated in the aqueous phase or lipid phase can attack erythrocyte membranes and induce a chain of lipids and proteins oxidation that trigger disruption in these membranes causing hemolysis [4]. Therefore, inhibition of oxidative damage by supplementation of antioxidants became an attractive therapeutic strategy to reduce risk of free radical mediated diseases [5].

Flavonoids, including the water soluble pigments anthocyanins are polyphenolic compounds which are widespread in fruits and beverages and possess a wide range of biological activities, of which anti-oxidation has been extensively explored [6]. Several *in vitro* studies indicated that anthocyanins are good antioxidants against free radicals initiated lipid peroxidation in solution [7], in micelles, human low

density lipoprotein (LDL) [8], and in liver microsomes [9]. Therefore, it is of interest to investigate the relationship between concentration-time and the possible anti-hemolytic activity of anthocyanins.

Azo compounds have been extensively used as a free radical initiators for biological and related studies, and the hemolysis induced by azo compounds provides a good approach for studying free radical-induced membrane damages [5, 10]. Both water soluble 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) and lipid soluble 2,2'-azobis(2,4-dimethyl)valeronitrile (AMVN) can sponta-neously decompose at physiological temperature in constant rate yielding, a known flux of carbon centered radicals that swiftly react with oxygen to yield peroxyl radicals to induce chain oxidation in erythrocyte membrane and red cell suspension [11]. The present study was designed to evaluate the concentration and time-dependent cytoprotective activity of anthocyanins against AAPH and AMVN-induced oxidative hemolysis of erythrocytes.

MATERIALS AND METHODS

Blood sampling and preparation of erythrocytes suspension

Peripheral venous blood samples were drawn from healthy non-smoker male donors**,** and collected into ethylenediaminetetraacetic acid, (EDTA) coated tubes. Erythrocytes (RBCs) were separated immediately by centrifugation of the collected blood at 4ºC and 3000 rpm for 10 min; plasma and buffy coat were carefully removed and discarded. RBCs were purified by three cycles of re-suspension and washing with isotonic phosphate buffer saline (PBS, pH 7.4). The isolated RBCs were used within one hour after their preparation, where 10% v/v erythrocytes suspension was prepared by suspending one volume of washed erythrocytes in nine volumes of isotonic PBS (pH 7.4).

Preparation of anthocyanins solution

Standardized powdered extract of anthocyanins (Mediolanum Pharma Ltd., Cournon d'Auvergne, France) of bilberry fruits (*Vaccinum myertillus*) was used in this study. Different concentrations of anthocyanins were prepared by dissolving the desired quantity of the powder in the same buffer used for preparation of erythrocytes suspension (pH 7.4) to get a stock solution, from which different dilutions were prepared to give concentrations of 1, 0.1 and 0.01 mg/ml of anthocyanins solutions.

Effect of different concentrations of anthocyanins on the oxidative hemolysis of erythrocytes induced by water and lipid soluble free radical initiators

The method is a modified procedure based on that established by Jimenez *et al*. [12]. Aliquots (1.5 ml) of freshly prepared erythrocytes suspension (10% v/v in PBS, pH 7.4) were pre-incubated for 30 min with each of the three different concentrations of anthocyanins (1, 0.1 and 0.01 mg/ml), followed by an additional 6 h period of incubation in absence or presence of free radical initiators [either 25 mM of AAPH or 10 mM of AMVN (Wako Chemical Ind., Osaka, Japan)] at 37ºC with constant and gentle shaking. Similarly other equal volumes of erythrocytes suspension were incubated with either PBS alone or each of the free radical initiators mentioned above, served as negative and positive control samples respectively. A 0.15 ml aliquot sample was removed from each incubation mixture at fixed time intervals to be diluted with PBS (pH 7.4), centrifuged at 3000 rpm for 5 min; the degree of hemolysis was assessed spectrophotometrically by recording the absorbance of the clear supernatant at 540 nm (Absorbance A). Correspondingly, another 0.15 ml was taken from erythrocytes suspension mixture and diluted with distilled water to yield complete hemolysis (100%); after centrifugation, the absorbance of supernatant was measured at 540 nm (Absorbance B) and was represented on the basis of the maximum absorbance in the aliquots of erythrocytes completely hemolyzed in distilled water;

Absorbance A / Absorbance B x 100 [13, 14]

Accordingly, the percentage of inhibition was calculated. The plotted data represent the mean of three independent experiments and the results were reproducible within 10% of standard deviation [14].

Effect of delayed addition of anthocyanins on the oxidative hemolysis induced by water and lipid soluble free radical initiators

The most effective concentration of anthocyanins (1 mg/ml) was added either concomitantly with the free radical initiators (AAPH or AMVN of 25 mM and 10 mM, respectively) or 2 and 3 h later after the addition of azo compounds to the incubation medium of the freshly prepared erythrocytes suspension (10% v/v in PBS, pH 7.4) at 37ºC. During the period of incubation, 0.15 ml aliquot sample was taken out from the reaction mixture at fixed time intervals and diluted with PBS (pH 7.4), centrifuged at 3000 rpm for 5 min; the level of oxy-Hb was determined by measuring the absorbance of supernatant at 540 nm (Absorbance A) using spectrophotometer. Reference values were determined by removing 0.15 ml of erythrocytes suspension from same incubation mixture and diluted with distilled water (100% hemolysis); after centrifugation absorbance of supernatant was measured spectrophotometrically at 540 nm (Absorbance B). The percentage of hemolysis was calculated by measuring the ratio of absorbance;

Absorbance A / Absorbance B x 100

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Also the percent of inhibition was determined [15]. The plotted data represent the mean of three independent experiments and the results were reproducible within 10% of standard deviation [14].

Statistical analysis

Data were presented as mean ± SD. All data were analyzed using analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

RESULTS

Effect of different concentrations of anthocyanins on the oxidative hemolysis induced by water and lipid soluble free radical initiators

In the present study, *in vitro* oxidative hemolysis of human erythrocytes was used as a model to study the oxidative damage of biological membranes induced by free radical generation at the extracellular site, and the possible protective effect of anthocyanins in this respect. In the control samples incubated in PBS without both anthocyanins and free radical generators (AAPH or AMVN) RBCs were stable and little hemolysis was reported, while after 6 h of incubation of RBCs suspension with either AAPH or AMVN the reported degree of hemolysis was approximately 44.8% and 96.5%, respectively. The hemolysis was maintained at the baseline level, similar to that in the control samples, when the erythrocytes suspension was incubated with increasing concentrations of anthocyanins (0.01, 0.1 and 1 mg/ml) without free radical initiators (Figs.1&2). Also it became evident that percent of hemolysis was decreased with increasing the concentration of anthocyanins solutions added to the incubation medium 30 min before the addition of either AAPH or AMVN to about 21.97, 10.6 and 5.9%, after 6 h of incubation of erythrocytes suspension with AAPH in presence of 0.01, 0.1 and 1 mg/ml anthocyanins, respectively (Fig.3); while 67.1, 51.2 and 17.6% hemolysis were achieved when AMVN was used instead of AAPH (Fig.4). The percent inhibition of erythrocyte hemolysis produced by anthocyanins in presence of either AAPH or AMVN was significantly increased with increasing the concentration of anthocyanins solution as shown in Fig.5.

Effect of delayed addition of anthocyanins on the oxidative hemolysis-induced by free radical generators

Addition of the most effective concentration of anthocyanins (1 mg/ml) to the incubation medium of erythrocytes suspension, concomitantly with or after 2 and 3 h from the addition of the free radical generators (AAPH or AMVN), caused a delay in the hemolysis induced by these chemicals as demonstrated in

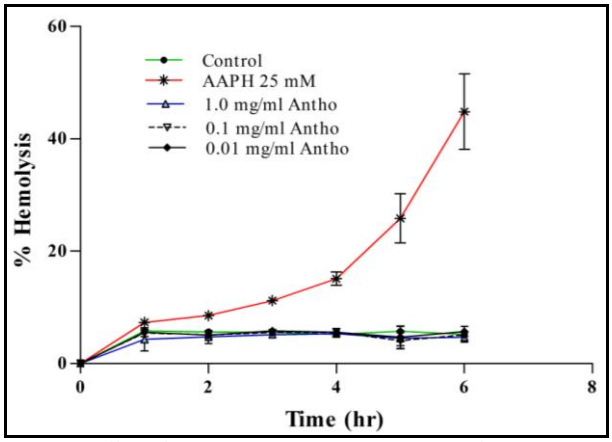

Figure 1. Effect of AAPH and 1, 0.1 and 0.01 mg/ml of anthocyanins alone on erythrocyte hemolysis. Data expressed as mean ± SD of three independent experiments.

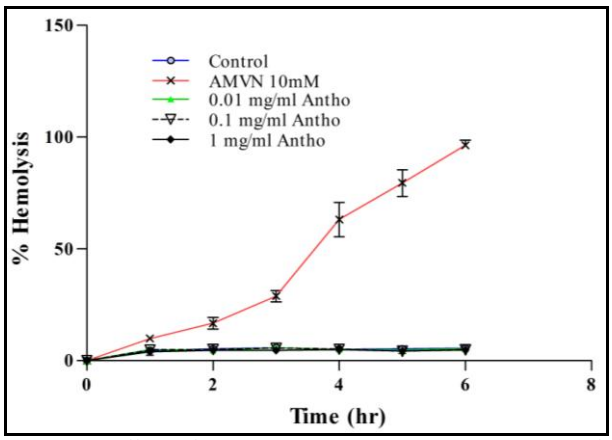

Figure 2. Effect of AMVN and 1, 0.1 and 0.01 mg/ml of anthocyanins alone on erythrocyte hemolysis. Data expressed as mean ± SD of three independent experiments.

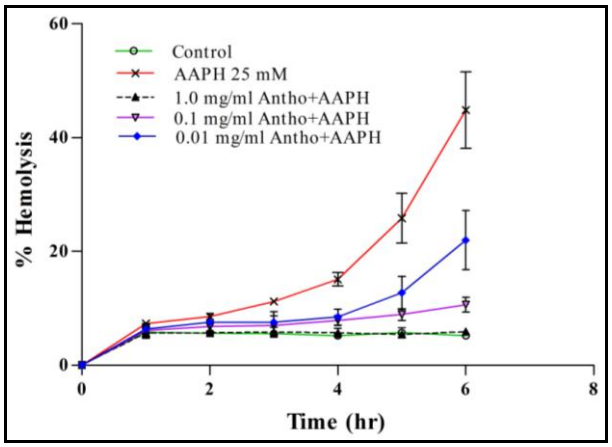

Figure 3. Effect of 1, 0.1 and 0.01 mg/ml of anthocyanins on oxidative hemolysis induced by AAPH in erythrocyte suspension. Data expressed as mean ± SD of three independent experiments.

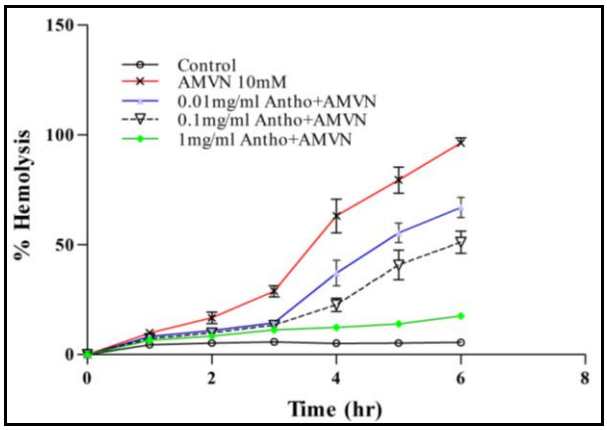

Figure 4. Effect of 1, 0.1 and 0.01 mg/ml of anthocyanins on oxidative hemolysis induced by AMVN in erythrocyte suspension. Data expressed as mean ± SD of three independent experiments.

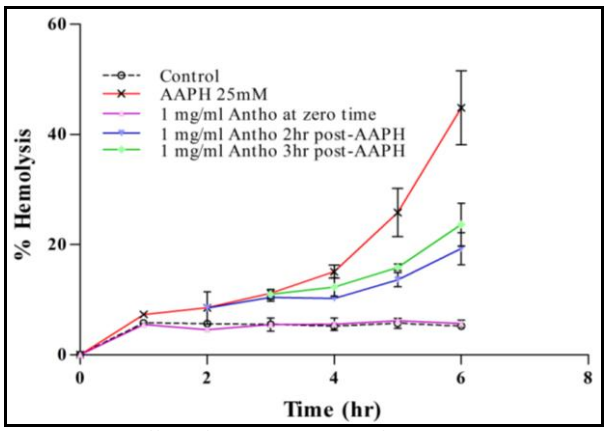

Figure 6. The effect of delayed addition of 1.0 mg/ml anthocyanins on oxidative hemolysis induced by AAPH. Data expressed as mean ± SD of three independent experiments.

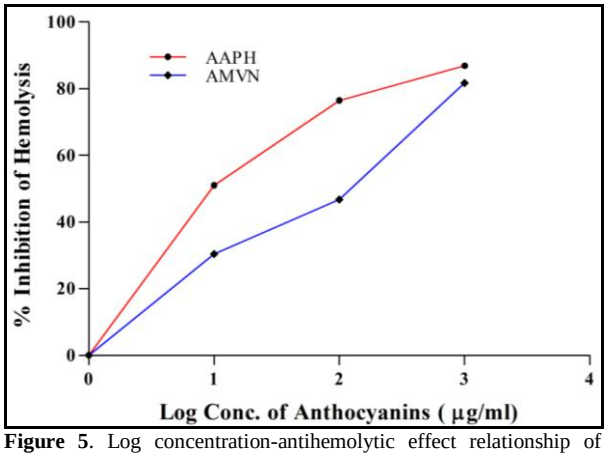

Figure 5. Log concentration-antihemolytic effect relationship of anthocyanins solutions (1, 0.1 and 0.01 mg/ml) in AAPH- and AMVN-induced oxidative hemolysis of erythrocytes *in vitro*. Data expressed as log concentrations of anthocyanins *vs* % inhibition of hemolysis.

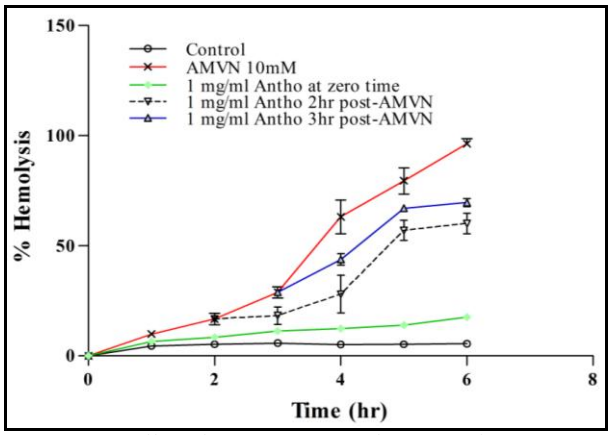

Figure 7. The effect of delayed addition of 1 mg/ml of anthocyanins on oxidative hemolysis induced by AMVN. Data expressed as mean ± SD of three independent experiments

Figs.6&7, revealed by the decrease in light absorbance at 540 nm which may be attributed to the relative decrease in the level of oxy-Hb released into the incubation medium. After 6 h of incubation, the percent hemolysis produced by 25 mM AAPH was 44.8% and addition of anthocyanins significantly reduce the hemolysis to 5.7, 19.3 and 23.6%, respectively, when added simultaneously or 2 and 3 h after the addition of AAPH (Fig.6). The percent hemolysis produced by AMVN was approximately 96.5%, which is considerably reduced to 17.9, 60.8 and 69.7% by the addition of anthocyanins at the same time or 2 and 3 h after the addition of AVMN (Fig.7). Estimation of percent inhibition produced by anthocyanins against free radical initiators AAPH- and AMVN-induced oxidative hemolysis was estimated, where 1 mg/ml anthocyanins added at zero time, 2 and 3 h after addition of AAPH produced 87.3, 57 and 47.3% inhibition, while in presence of AMVN about 81.4, 36.9 and 27.8% inhibition was reported.

DISCUSSION

Free radicals attack erythrocyte membrane components, such as proteins and lipids and cause changes in structure and function of membranes, which may result in hemolysis [16]. Erythrocytes are particularly sensitive to oxidative damage due to the presence of high polyunsaturated fatty acids content in their membranes and high cellular concentrations of oxygen and Hb, a powerful catalyst which promote erythrocyte oxidative stress; therefore, RBCs are often considered as a convenient model to study membrane oxidative damage induced by various xenobiotics/pro-oxidants [17]. Maintaining normal RBC architecture should be an important therapeutic approach in some pathological conditions associated with hematological disorders [18]. Recently, overwhelming evidence has supported the hypothesis that nutritional sources containing flavonoid antioxidants might have beneficial role in retarding, or even reversing the course of several disorders induced by oxidant challenge [19].

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Anthocyanins, as bioactive components of these dietary sources, could play a major role in enhancing the biological antioxidant system [20] as long as they have antioxidant capacity [21]. Anthocyanins were reported to inhibit lipid peroxidation in several *in vitro* lipid containing models such as liposomal membrane, liver microsomal system and human LDL exposed to oxidative damage; this protection was explained by the ability of anthocyanins to scavenge free radicals and at the same time spare other antioxidants from oxidation [22]. Recently, interesting data have been reported indicating the protective effect of flavonoids against oxidative damage in intact RBC membrane and RBC ghost [23, 24]. In many cases, the bioactivity of flavonoids was found to be correlated with their ability to interact with cell membrane [25, 26]; accordingly, they protect membrane lipids and proteins against free radical-induced injury [27].

Membrane interactions of flavonoids are connected with modification of the physico-chemical and thermodynamic properties of the membrane [28]. Flavonoids can modify membrane permeability and membrane dependent processes, change fatty acid composition or phospholipid content in membrane or interact with membrane proteins. The previously mentioned cellular changes induced by flavonoids may be crucial for their pharmacological activity employed in the treatment of certain diseases. Other results indicated the possible incorporation of anthocyanins into plasma membrane of endothelial cells, where they positively affect membrane stability, permeability and reducing fragility of vascular tissue [29]; also other *in vitro* cell models showed the ability of anthocyanins to localize within cell membrane of the vascular endothelium conferring significant protective effects against oxidative insult [30].

From all these studies we can predict that anthocyanins may also be able to localize within the membrane milieu of red blood cells. Therefore, in the present work we studied the ability of different concentrations of anthocyanins to protect the membrane components (lipid/proteins) against the oxidative damage initiated by both water and lipid soluble free radical generators (AAPH and AMVN). Using this model, the contribution of intracellular metabolic process in the generation of free radicals and hemolysis was excluded; also we can assess the anti-hemolytic effect of anthocyanins against external aggressors and distinguish their actions toward aqueous and lipophilic radicals separately. Water soluble azo compound (AAPH) generates radicals in hydrophilic domain of membrane lipid bilayers, while the lipid soluble azo compound (AMVN) usually acts in the hydrophobic domain; both decompose at 37ºC at a constant rate generating peroxyl radicals in the presence of oxygen. The generated peroxyl radicals then induce chain oxidation of proteins and lipids in erythrocyte membrane components and eventually cause changes in structure and function of membranes [31].

The results of the present study showed time-dependent erythrocyte hemolysis during cell incubation with either AAPH or AMVN, indicating peroxidative damage; while hemolysis was maintained at baseline level similar to that of control sample when erythrocytes incubated with different concentrations of anthocyanins alone, which signify the absence of abnormal lipid oxidation as demonstrated in Figs.1&2. Also we reported that hemolysis started after 2 h in erythrocytes exposed to AAPH, while higher extent and less time was needed to induce hemolysis in cells exposed to AMVN; this may be related to increased lipid peroxidation as previously observed by others, who reported direct correlation between hemolysis and increased lipid peroxidation in erythrocyte membrane, since AMVN can attack the lipid core of the membrane, where radicals can diffuse easily resulting in rapid disruption of membrane bilayers [32]. The observed lag period extended between 1 and 2 h before hemolysis could be attributed to the function of endogenous enzymatic and non-enzymatic antioxidant systems in erythrocytes, which can efficiently quench radicals and consequently reduce hemolysis at early time [33].

When production of peroxyl radicals and other reactive species formed throughout chain propagation overrides the antioxidant barriers, an oxidative stress condition will develop, including damage to the erythrocyte membrane which may lead ultimately to hemolysis [11]. It is clearly evident in Figs.3&4 that anthocyanins protected erythrocyte membrane from oxidative hemolysis induced by both AAPH and AMVN in concentration dependent manner, as there is direct correlation between the lag time and anthocyanin concentration as well as the percent inhibition of hemolysis, increased by increasing the concentration of anthocyanins (Fig.5).

It has been reported that peroxyl radicals generated by AAPH are effective in promoting modifications in band-3 protein located in the lipid bilayers of erythrocytes and to less extent affect spectrin located on the cytosolic side of the membrane, but they are unable to promote oxidation of intracellular Hb suggesting a lack of accessibility of AAPH to the intact erythrocyte. Additionally, AAPH radical do not induce significant lipid peroxidation of human erythrocyte, indicating that peroxyl radicals were unable to gain access to the polyunsaturated fatty acids localized on the cytosolic face of the erythrocyte membrane [34]. AAPH was suggested to induce hemolysis via the formation of membrane pores and band-3 oxidation,

indicated that erythrocyte protein oxidation, possibly in the hydrophobic core, plays a significant role in the membrane pre-hemolytic damage and it has been proposed that peroxidized lipids around band-3 protein may promote its redistribution [11]. In this line, Celedon *et al* [35] reported that membrane protein degradation constituted a major event associated with the AAPH-induced lysis of erythrocyte. On the other hand, AMVN act on the hydrophobic core of the membrane to induce lipid peroxidation and increased plasma membrane fluidity that enhance rapid diffusion of peroxyl radical through the membrane [36].

The data of the present study revealed that anthocyanins display concentration and time dependent anti-hemolytic effect against peroxyl radicals generated by both AAPH and AMVN; this protection involved both hydrophilic and lipophilic domains of the erythrocyte membrane, which could be attributed to the antioxidant activity of anthocyanins that decrease the formation of free radicals generated by azo compounds, since anthocyanins contain one or more aromatic hydroxyl groups, being responsible for the antioxidant activity, that actively scavenge free radicals. So, presence of anthocyanins in the incubation medium of RBCs may quench the chain propagating peroxyl radicals, hence reducing the extent of peroxidation and attenuate hemolysis. Moreover, they may spare, maintain, or regenerate endogenous antioxidants like GSH, ascorbate and α-tocopherol by donating hydrogen to the oxidized forms of these molecules, so they can protect membrane lipids from peroxidative damage induced by AMVN [37].

Furthermore, it was known that polyphenols enhance red blood cell resistance to oxidative stress both *in vitro* and *in vivo* [38]. Recent studies have suggested the ability of certain polyphenols to partition in cell membranes, and the resulting restriction of their fluidity could sterically hinder diffusion of free radicals, thereby decrease the kinetics of free radical reactions [39]. Actually, interaction and localization of flavonoids within cell membrane greatly related to their lipophilicity that affected by chemical structure and polarity (number of un-substituted hydroxyl groups), which result in two possible relevant interactions between flavonoids and lipid bilayers: *(i)* adsorption on the membrane surface through interaction of the hydrophilic flavonoids with the polar head groups of phospholipids at the water/lipid interface, mainly associated with the formation of hydrogen bonds; this type of interaction may provide certain level of protection for membrane bilayers against external and internal oxidants; and *(ii)* partitioning of the flavonoid in the non-polar core of the membrane, associated with its hydrophobic interaction with the fatty acyl chain and result in chain breaking antioxidant activity [40].

The type of interactions of flavonoids with membranes may be considered as a contributing factor that determines their antioxidant capacity. Furthermore, as recently shown, flavonoid-membrane surface interactions could protect the integrity of lipid bilayers from disrupting agents [41]. Depending on these findings, we expect that the hydrophilic nature of anthocyanins enhance their interaction at the water/lipid interface, affecting membrane fluidity and sterically hinder diffusion of free radicals into the membrane, thereby decrease the rate of peroxidation and free radical reactions as observed previously [36]. In the present study, we observed that anthocyanins more effectively inhibit AAPH-induced hemolysis than that of AVMN indicating that the potential capacity of anthocyanins to protect membrane proteins exceeds its ability to interfere with the membrane lipid peroxidation. This could be explained by the efficiency of anthocyanins as hydrophilic flavonoids for trapping radicals generated in the aqueous media by AAPH, while the interaction and localization in the membrane would be more relevant when radicals generated in the lipid phase by AMVN. The interpretation that anthocyanins trap initiator radicals is in line with the early observation by Niki *et al* [32] that uric and ascorbic acids, two highly water soluble molecules, promote their cytoprotective effects by scavenging AAPH-derived peroxyl radicals. Also, the possible interaction of anthocyanins with membrane proteins may shield their attack by peroxyl radicals and enhance the protection afforded against AAPH, through protection of membrane lipids and proteins [26, 27].

The present study also indicated that delayed addition of anthocyanins two and three hrs after the addition of the azo compounds resulted in noticeable reduction in their anti-hemolytic and cytoprotective effects (Figs.6&7), which appeared to be higher with AMVN than AAPH. These results suggested that within the elapsed time, AAPH and AMVN have already triggered oxidative modifications that can no longer be prevented or reversed by the presence of anthocyanins, in addition to the rapid kinetic of lipid peroxidation, which increases the accessibility of lipophilic radicals into the cell with consequent initiation of intracellular oxidation of Hb, a powerful catalyst of lipid peroxidation process.

Since leakage of Hb into the extracellular medium represents the only final event to measure the extent of hemolysis, it is not possible from the present data to determine the exact mechanism by which anthocyanins exerted their cytoprotective effects. However, given the possibility that oxidation of membrane lipids and/or proteins represents significant events underlying erythrocyte lyses, our results indicated that anthocyanins provided acceptable level of protection

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for both membrane components (hydrophilic and lipophilic) in a concentration and time dependent pattern. In conclusion, anthocyanins protect RBCs against the plasma membrane damage induced by AMVN and AAPH in a concentration and time dependent pattern.

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