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The Concentration-Antioxidant Effect Relationship of Anthocyanins on the Time Course of Nitrite-induced Oxidation of Hemoglobin: *In vitro* study

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Abstract: Background: Phytochemicals such as anthocyanin are gaining great importance because of their contribution to human health as potent antioxidants with radical scavenging and cytoprotective effects. The present study was designed to evaluate the concentration-antioxidant relationship of anthocyanins using *in vitro* model of nitrite-induced hemoglobin oxidation. **Methods:** Blood samples were collected from healthy volunteers for the preparation of erythrocyte hemolysate. Different concentrations of anthocyanin (10^{-12}-1.0 mg mL^{-1}) were incubated concomitantly with 1 mL of sodium nitrite (final concentration 1 mM) and the formation of met-Hb was monitored spectrophotometerically at 631 nm each min for 35 min compared with sample without anthocyanin (control). **Results:** Nitrite-induced rapid oxidation of Hb to met-Hb in the absence of anthocyanins, while oxidation process was delayed in a concentration dependent manner in presence of different concentrations of anthocyanins according to the time required to induce 50% oxidation of Hb relative to control. Anthocyanin also has protective effect when added before, together and early after the addition of nitrite. **Conclusion:** In conclusion, anthocyanin protects hemoglobin against oxidative reaction induced by nitrite in time and concentration depended manner and such protection found to be extended over long period of time.

Key words: Anthocyanins, RBCs, oxidative stress, Hb oxidation

INTRODUCTION

Reactive Oxygen Species (ROS) including super oxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxyl radicals (R-OO˙) are of increasing interest as agents of different pathologic states including atherosclerosis, inflammatory arthritis, diabetes, cardiovascular disorders and neurodegenerative diseases (Durak *et al*., 2001; Buyukkocak *et al*., 2000). Despite lack of mitochondria in erythrocytes, ROS are continuously produced in these cells due to high oxygen tension in arterial blood and their abundant heme iron contents (Johnson *et al*., 2005). Hemoglobin (Hb), the oxygen carrier protein, is the major source of ROS in erythrocytes, as it continuously generate O_2^- during Hb auto-oxidation process (Pandey and Rizi, 2010). Although oxidative stress may damage the red cell itself, the mass effect of large quantities of ROS leaving the red cell have tremendous potential to damage other components of the circulation (Scalbert *et al*., 2005). There is an overwhelming evidence to suggest that nutritional sources of antioxidants attenuate tissue damage caused by oxidative challenge (Rizvi *et al*., 2005), these nutritional sources could play a major role in reducing the incidence of many free radicals-mediated disorders (Rizvi *et al*., 2005). Anthocyanins are natural colorant belong to flavonoid subgroup of dietary polyphenols and proved to have potent antioxidant properties (Wang *et al*., 1997; Mazza *et al*., 2002). Anthocyanins have many therapeutic effects in wide range of diseases including anti-inflammatory (Youdim *et al*., 2002; Wang and Mzza, 2002), neuroprotective action (Youdim *et al*., 2000a; Galli *et al*., 2002) and reduce the risk of coronary heart disease through vasoprotective activities and inhibition of platelet aggeregation (Erlund *et al*., 2008). In addition to that, numerous studies also report their capacity to reduce general markers of oxidative stress (Kahkonen and Heinonin, 2003), including protection of RBCs against free radical since they have a potent scavenging activity (Blasa *et al*., 2007). According to our knowledge, there are no studies which clarify the exact relationship between concentration and their antioxidant activities of anthocyanins. Therefore, this study was designed to investigate the antioxidant activity of anthocyanins in different concentrations using in vitro model of nitrite-induced oxidation of hemoglobin in hemolysate.

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MATERIALS AND METHODS

Preparation of hemolysate: Blood samples were collected from healthy individuals in EDTA-containing tubes and centrifuged at 3000 rpm and 4°C for 10 min to remove plasma and the buffy coat of white cells. Then the packed erythrocytes were washed thrice with phosphate buffer saline (PBS pH 7.4) and lysed by suspending in 20 volumes of 20 mM phosphate buffer (pH 7.4).

Preparation of anthocyanin solutions: Different concentrations of anthocyanins (Mediolanum Pharma Ltd, France) were prepared by dissolving the required quantity in phosphate buffer (pH 7.4) to prepare stock solution (1 mg mL^{-1}) from which serial dilutions were prepared to give concentrations of 0.1, 0.01, 10^{-3}, 10^{-6}, 10^{-9} and 10^{-12} mg mL^{-1} Anthocyanin solutions.

Effect of different concentrations of anthocyanins on the time course of nitrite-induced oxidation of hemoglobin: *In vitro* model for oxidation of hemoglobin with sodium nitrite was utilized for production of methemoglobin (met-Hb) (Doyle *et al*., 1982). To 1 mL of freshly prepared hemolysate, 1 mL of different concentrations of anthocyanins (1, 0.1, 0.01, 10^{-3}, 10^{-6}, $10^{-9}$and 10^{-12} mg mL^{-1}) each time were added concomitantly with 1 mL^{-1} of sodium nitrite (BDH Ltd., Poole, England) (final concentration 1 mM) and the formation of met-Hb was monitored spectrophotometrically at 631 nm each minute for 35 min using UV-visible spectrophotometer (Specord-40, Germany). In other set of experiment, to 1 mL^{-1} of freshly prepared hemolysate 1 mL of the most effective concentration of anthocyanins was added either 10 min before or at 5 and 10 min after the addition of sodium nitrite. The formation of met-Hb was monitored as previously mentioned. Control experiments were conducted without anthocyanins and all experiments were performed in triplicate. The results were accepted with less than 10% SD.

RESULTS

Sodium nitrite causes rapid oxidation of hemoglobin to Met-Hb as demonstrated by the control curve, in which the oxidation process shows two stages, a slow initial stage followed by a rapid autocatalytic stage which bring the reaction to completion; whereas in presence of different concentrations of anthocyanins, the oxidation process was delayed in a concentration dependent manner as shown in (Fig. 1, Table 1). The time required to produce 50% oxidation of hemoglobin in erythrocyte hemolysate of control was 9 min, this time increased directly with increasing the concentration of anthocyanins (Table 2). Addition of the highly effective concentration of anthocyanin (1 mg mL^{-1}), 10 min before nitrite, as well as, 5 and 10 min after nitrite addition (i.e., during autocatalytic phase) decreases the absorbance of light attributed to Met-Hb formation which is considered as an index for protection against Hb oxidation with sodium nitrite as shown in Fig. 2 and the time required to convert 50% of the available hemoglobin

Table 1: The time required by each concentration of anthocyanins to produce 50% oxidation of Hb by sodium nitrite

Anthocyanin concentrations mg mL^{-1}	Time to form 50% Met-Hb
Control	9.0±4.2
10^{-12}	16.1±1.7
10^{-9}	17.6±1.5
10^{-6}	21.0±2.6
10^{-3}	26.6±2.5
10^{-2}	45.0±7.0
0.1	86.3±4.5
1	122.6±5.5

The results represent Mean±SD of 5 experiments

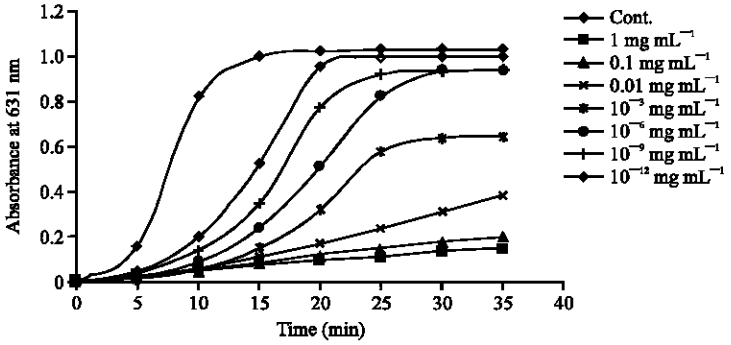

Fig. 1: Effect of different anthocyanin concentrations on the time-course of nitrite-induced Hb oxidation in erythrocyte lysate

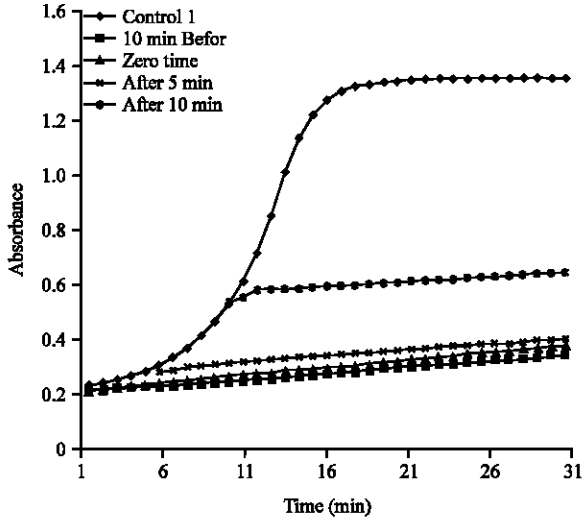

Fig. 2: Effect of 1 mg mL^{-1} anthocyanin on the time course of Met-Hb formation when added at different time intervals of adding nitrite to erythrocyte lysate

Table 2: Effect of different anthocyanin concentrations on the formation of Met-Hb in erythrocyte lysate

Anthocyanin concentrations (mg mL^{-1})	% Formation of Met-Hb	% Inhibition of Met-Hb
Control	100	0
10^{-12}	96.26	3.74
10^{-9}	91.2	8.8
10^{-6}	90.49	9.51
10^{-3}	62.24	37.76
10^{-2}	37.09	62.91
0.1	19.72	80.28
1	14.01	85.99

Results represent the mean of 3 experiments; the control was treated with nitrite alone

Table 3: Effect of 1 mg mL^{-1} anthocyanin on the time course of 50% Met-Hb formation at different time intervals of adding nitrite to erythrocyte lysate

Time for the addition of 1 mg mL^{-1} anthocyanins	% Formation of Met-Hb	% Inhibition of Met-Hb	Time to form 50% Met-Hb (min)
Control	100	0	9
Before 10 min	12.4	87.6	141
At Zero time	14.34	85.66	122
After 5 min	16.79	83.21	104
After 10 min	39.49	60.51	45

Results represent the mean of 3 experiments

to Met-Hb was prolonged when anthocyanin added 10 min before and 5 or 10 min after the addition of sodium nitrite to the hemolysate (Table 3).

DISCUSSION

Nitrite was a commonly known pro-oxidant to Hb and involved in the generation of nitrate and Met-Hb (Titov and Petrenko, 2005). The kinetic of this reaction, when nitrite in excess, are complex and exhibit an initial slow phase (lag phase) that accelerates into a rapid phase (propagated phase) of oxidation. This reaction profile has been modeled as an autocatalytic, free radical chain reaction (Keszler *et al*., 2008). Several reactive oxygen and nitrogen species in addition to intermediate protein-free radicals are involved in the formation of Met-Hb including superoxide anion, hydrogen peroxide, nitrogen dioxide and ferryl heme radicals (Winterbourn, 1985). In the present study, it has been reported that anthocyanins, isolated from bilberries, were able to ameliorate the experimentally induced Hb-oxidation by nitrite and formation of ROS in RBCs using *in vitro* model. This finding would suggest a potential beneficial role that may arise following dietary consumption of bilberries. Indeed, a large number of studies have reported various beneficial physiological effects in wide range of free radical mediated disorders that polyphenols may elicit as demonstrated in *in vitro* model systems. Some examples includes; modulation of cell signaling (Gopalakrishnan *et al*., 2006) protection of DNA integrity (Sestili *et al*., 1998); alteration of immune and inflammatory responses (Gonzalez-Gallego *et al*., 2007; Karlsen *et al*., 2007) and modification in cytokine production (Garcia-Alonso *et al*., 2009; Xia *et al*., 2007). Moreover, other studies reported enhanced protection afforded to RBCs by polyphenolics (Youdim *et al*., 2000b; Zhu *et al*., 2002). In this regard, our *in vitro* findings support supported those reported by others concerning the time and concentration-dependent protection of Hb by anthocyanins against oxidative effect of sodium nitrite, as they delay the onset of autocatalytic stage of this reaction, a mechanism which seems to be associated with reduction of oxidative stress at the cellular level and mostly can be explained by the antioxidant capacity of anthocyanins (Silva *et al*., 2002). This effect can be mediated by several ways including proton donation and/or scavenging property to neutralize the action of several reactive intermediates that promote continuous conversion of oxy-Hb to Met-Hb, as superoxide and ferryl hemoglobin radicals (Wang and Stoner, 2008; Gebicka and Banasiak, 2009); this protective effect is largely related to the chelating effects of iron by polyphenols (El-Hajji *et al*., 2006) which can catalyze the formation of OH by fenton reaction as long as H_2O_2 and O_2^- are existing in the reaction media (Cimen, 2008). Also the result of this study suggests that the protective effect of anthocyanin is not mediated by reduction of Met-Hb, since it fails to reverse back the oxidized Hb when added at later stages; in addition, the direct interaction between nitrite and anthocyanins as a reason for protection is

ruled out because the concentration of anthocyanin that produce protection is very low compared to that of nitrite. The ferryl derivatives of Hb are products of the reaction of oxy^{-} and Met-Hb with H_2O_2. Ferryl Hb, either with or without a radical site on protein moiety, are oxidizing species toward globin moiety that finally lead to formation of hemichrome and dimmers of Hb subunits are also formed(Giulivi and Davies, 1994; Kowalczyk *et al*., 2007). The reduction of the ferryl form of Hb is facilitated by some reducing compounds, including ascorbate, rutin, urate, melatonin and epigallocatechine (Tesoriere *et al*., 2001; Jia and Alayash, 2008), this may indicate that inactivation of ferryl Hb species is necessary for protection. So, the beneficial effect of anthocyanins against oxidative damage of Hb could be in part mediated by the ability of anthocyanins to neutralize ferryl Hb reactive species (Gebicka and Banasiak, 2009). In conclusion, anthocyanins protect Hb from oxidative damage induced by sodium nitrite *in vitro* and delay the formation of Met-Hb in a concentration and time dependent manner.

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