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Research article

PROTECTIVE EFFECT OF PLACENTA EXTRACTS AGAINST NITRITE-INDUCED OXIDATIVE STRESS IN HUMAN ERYTHROCYTES [#]

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Abstract: Aqueous-saline human placenta extract (HPE) is known to possess antioxidant activity due to the high concentration of bioactive substances. This fact allows its application in clinical practice in order to treat oxidation-induced diseases. Extract antioxidant activity is mainly conditioned by proteins. Freezing of extracts has been shown to lead to their antioxidant activity increasing due to protein conformation changes.
Different biological models are widely used in order to evaluate efficacy of novel antioxidants and mechanisms of their action. One such model appears to be erythrocytes under nitrite-induced oxidative stress. Nitrite is known to be able to penetrate erythrocyte membrane and to oxidize hemoglobin. In order to investigate whether HPE is able to decrease nitrite-induced oxidative injuries and to evaluate the protein contribution to this process, spectrophotometric and electron spin resonance (ESR) assays were used.
Experimental data have revealed that antioxidant activity of extracts and of some of their fractions correlates with methemoglobin concentration lowering. Preliminary erythrocyte incubation with an extract fraction of 12 kDa allows preservation of the structural-dynamic cytosol state the closest to the control.

Key words: Human placenta extract, Protein, Antioxidant activity, Oxidative stress, Nitrite, Erythrocyte, Methemoglobin, Cytosol, Freezing, Thawing

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Abbreviations used: ESR – electron spin resonance; ABTS – 2,2'-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid); HPE – human placenta extract; RBC – red blood cell

INTRODUCTION

Antioxidants are known to be inhibitors of oxidation processes, possessing different structure and mechanisms of pro-oxidant scavenging. They can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers [1, 2, 3]. Different biological models are widely used in order to evaluate efficacy of novel antioxidants and mechanisms of their action [3]. One such model appears to be erythrocytes under nitrite-induced oxidative stress. Nitrite is known to be able to penetrate erythrocyte membrane and, when in high extracellular concentrations, to oxidize hemoglobin. Nitrite uptake was reported to be sensitive to temperature, pH and ionic composition of the medium. About 25% of nitrite uptake occurred on the sodium-dependent phosphate transporter and the rest as diffusion of nitrous acid or other species across the plasma membrane at pH 7.4 [4]. Hyperpolarization of erythrocyte membranes and an increase in membrane rigidity have been shown as a result of RBC oxidation by sodium nitrite. These membrane changes preceded reduced glutathione depletion and were observed simultaneously with methemoglobin (metHb) formation. Methemoglobin formation has been demonstrated to be increased in proportion to the intracellular nitrite concentration [5]. The influence of nitrite on other RBC properties has not been fully investigated.

Analysis of human placenta extracts (HPEs) has revealed that such extracts appear to possess antioxidant activity. Thus HPEs have been shown to scavenge hydroxyl radical, nitric oxide, and superoxide radical; to reduce ferric iron; to chelate transition metal ions; and to prevent lipid peroxidation [6, 7]. Such activity may be explained by the presence of antioxidants able to be donors of electrons such as phenol compounds, ascorbic acid, uric acid, some SH-group-containing compounds and protein amino acids [8]. These facts support HPE application in clinical practice in order to treat oxidation-induced diseases including atherosclerosis, aging, inflammation, and certain nervous system disorders.

In our work we investigated whether HPE is able to decrease nitrite-induced oxidative injuries in erythrocytes. We also tried to evaluate the protein contribution to this process.

MATERIALS AND METHODS

Human red blood cells (RBCs) were obtained from the whole donor blood by centrifuging and washing out with physiological solution. Then erythrocytes were diluted in a 1:1 ratio with 5 mM phosphate-buffered saline, pH 7.4. Methemoglobin content in erythrocytes varies within the range from 0.8 to 1.2%. In the research there were used 8 placentae, derived from parturients with their informed consent (38 weeks' gestation term). Aqueous-saline placenta extracts were obtained from homogenate of placenta separated from conjunctive tissues by 12-h exposure to physiological solution at 4ºC. Protein concentration in HPE varies from 8 to 10 mg/ml.

Nucleotide concentration was 1.2-1.4 mg/ml. In our work we have used extracts with no hemolytic activity. The obtained extracts were frozen down to -196ºC at the rate of 300º/min in a Dewar flask with liquid nitrogen. Extracts were thawed in a water bath at 20ºC. Separate fractions of extracts were obtained using the gel chromatography method on a 21x2 cm column with Sephadex G-200. HPE fractions were standardized by protein concentration. Maximum protein concentration in the fraction with 100-60 kDa fractions was 1.8 mg/ml. About 60% of nucleotide content was in fractions with molecular mass less then 5 kDa. During experiments erythromass was associated with an equal volume of HPE or its separate fractions.

Spectrophotometric studies of antioxidant activity of HPEs and their fractions were carried out according to the method of Re et al. [12]. $ABTS^+$ cation radical was produced by the reaction between 7 mM ABTS (*2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid*) in H_2O and 2.45 mM potassium persulfate, stored for 12 h in darkness at room temperature. The initial substance (0.1 ml) was diluted with 3 ml of distilled water. $ABTS^+$ (0.1 ml) was added to the experimental sample and the kinetics of $ABTS^+$ radical solution decolorization was recorded. Results are presented as percentage changes in absorbance of initial $ABTS^+$ solution.

In order to investigate the influence of nitrite on erythrocytes and to find out if HPEs possess any protective effect against nitrite-induced oxidation, the samples were divided into three parts. The first one was erythrocytes after incubation for 20 min with nitrite solution, which did not lead to erythrocyte hemolysis but to partial oxidation of intracellular hemoglobin. The second one was erythrocytes after simultaneous incubation with sodium nitrite and HPE (fresh or frozen-thawed) or its separate fractions. The last one contained erythrocytes which were preliminarily incubated for 2 hours with HPE (fresh or frozen-thawed) or its separate fractions, then were washed out and incubated with sodium nitrite for 20 min. Sodium nitrite was dissolved in 5 mM phosphate buffer saline. All experimental samples were washed out of the nitrite-containing medium and methemoglobin content in cells was evaluated spectrophotometrically [13] (using Pye Unicam SP8000 spectrophotometer).

We also measured the level of autohemolysis, and kinetics of low-pH hemolysis in citrate-phosphate buffer with pH 3.8 [14]. Osmotic fragility was evaluated by the level of hemolysis of RBC in a series of hypotonic saline solutions of decreasing ionic strength.

In order to estimate the potential of the methemoglobin reducing system, experimental samples were washed out of nitrite containing solutions and methemoglobin concentration alterations were investigated during storage at 4ºC for 2 days. Hemolysis of erythrocytes during this experiment was not registered.

Erythrocyte cytosol state was characterized by the ESR method using TEMPON [15]. Such a method allows microviscosity and barrier membrane properties to be assessed. Temperature dependence of the TEMPON mobility parameter characterizes RBC membrane and cytosol components which are involved in

temperature-induced structural alterations. All experiments were repeated at least three times. Figures present means ± standard error of means. The statistical significance of differences was estimated by Student's t-tests. The P-value < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

In our experiments we used an extracellular concentration within the range of 10-50 mM. Erythrocyte methemoglobin concentration was found to correlate with nitrite concentration (Fig. 1A). At the same time, osmotic erythrocyte stability did not change significantly (Fig. 1B). The time of 50% low-pH hemolysis was found to decrease but not to be dependent on the nitrite concentration (Fig. 1C). In the rest of our experiments we used the concentration of 10 mM, under which intracellular methemoglobin concentration was 50-60% depending on the donor. Erythrocyte cytosol microviscosity, measured with the ESR method, under such a nitrite concentration was found to decrease significantly in comparison with the control (Fig. 1D).

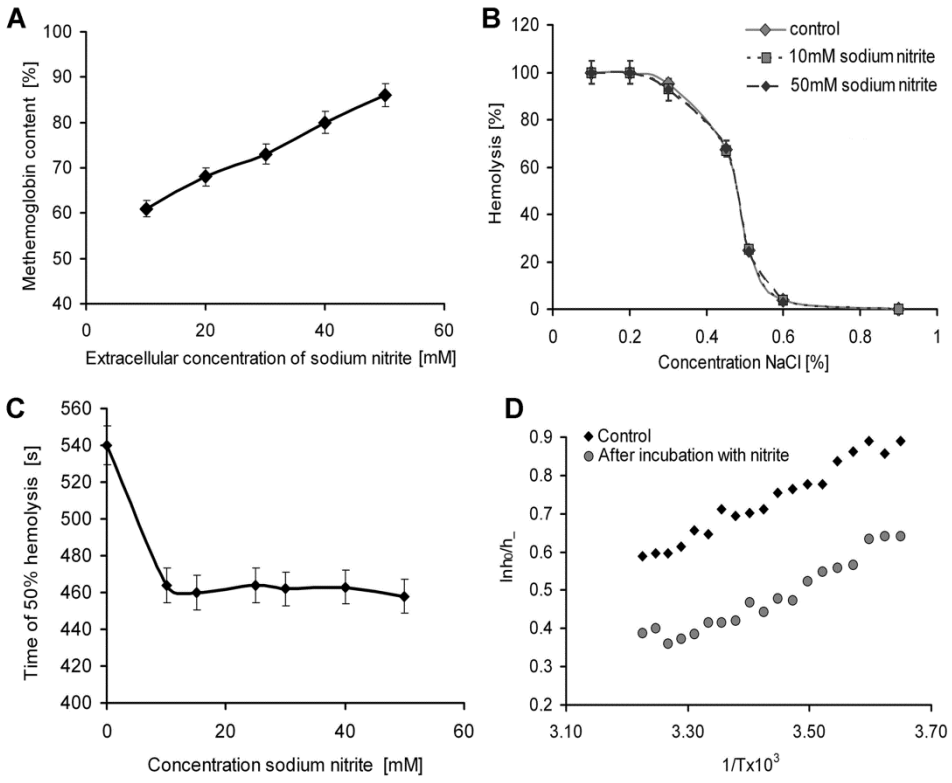

Fig. 1. Erythrocytes under nitrite-induced oxidative stress. A – methemoglobin content in erythrocytes after incubation with nitrite and following washing out; B – RBC osmotic fragility; C – RBC low-pH stability; D – influence of incubation with nitrite (10 mM) with subsequent washing out on RBC cytosol microviscosity.

Simultaneous incubation with nitrite and HPE or its separate fractions allows erythrocyte methemoglobin concentration to be lowered (Fig. 2). It should be mentioned that almost all HPE fractions provided an antioxidant action. In order to assess antioxidant properties of HPEs and separate fractions, we used the method based on the ability to inhibit the $ABTS^+$ radical. Kinetics of $ABTS^+$ radical cation reduction measured as the percentage inhibition of absorbance at 734 nm includes 2 phases due to the presence of rapid- and slow-reducing antioxidants [9, 16]. Activity of antioxidants responsible for rapid reduction (such as ascorbic acid, uric acid, α-tocopherol, amino acids containing SH groups, reduced glutathione, phenol compounds and ubiquinones) was evaluated by decolorization during the first 10 s. Activity of antioxidants responsible for slow reduction was estimated by decay during the next 390 s (Fig. 3A).

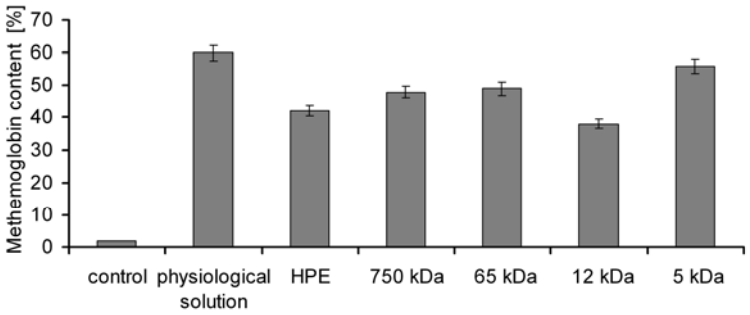

Fig. 2. Influence of simultaneous incubation with nitrite and HPE or its separate fractions on methemoglobin formation in RBC.

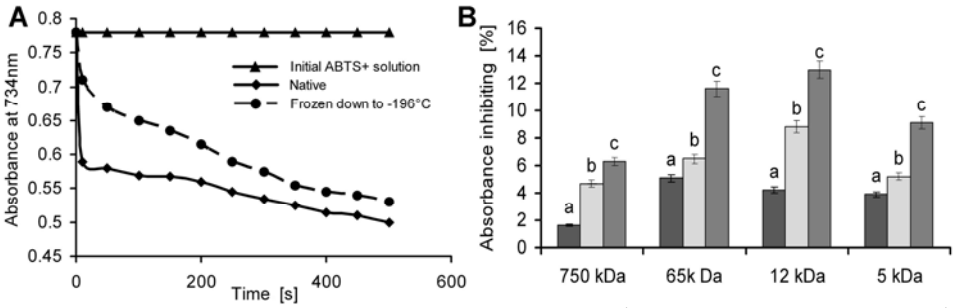

Fig. 3. Antioxidant activity of HPEs. A – kinetics of $ABTS^+$ radical reduction; B – $ABTS^+$ radical reducing activity of HPE fractions: a – rapid reduction, b – slow reduction, c – total reduction.

Ability of extract fractions with molecular mass over 5 kDa to decrease methemoglobin concentration correlates with their antioxidant activity provided by slow reducing centers (Fig. 3B). We found the fraction with molecular mass of 12 kDa to be the most effective. Obtained data constitute evidence of extract proteins' important role in erythrocyte protection against nitrite-induced oxidative stress. We have also investigated the influence of freezing down to

-196°C on HPE antioxidant properties. Freezing was found to lead to lowering of rapid reducing centers' activity and to increased activity of slow ones (Fig. 3A). Our experiments with frozen-thawed extracts have shown that their effectiveness in lowering the influence of nitrite on erythrocytes was higher than that of fresh HPE. The slow phase of $ABTS^{+}$ reduction is known to be preconditioned by proteins. Previously reported data have shown that the freeze-thawing process results in protein conformational changes leading to molecule loosening [17, 18]. These facts and obtained results confirm our assumption about proteins' leading role in antioxidant HPE action. Thus we believe that the observed alterations occur due to protein conformational changes connected with increased availability of protein antioxidant centers.

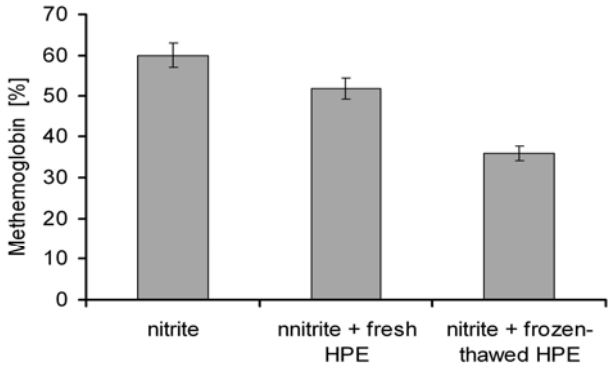

Fig. 4. Influence of freezing-thawing on HPEs' ability to reduce methemoglobin content in RBC.

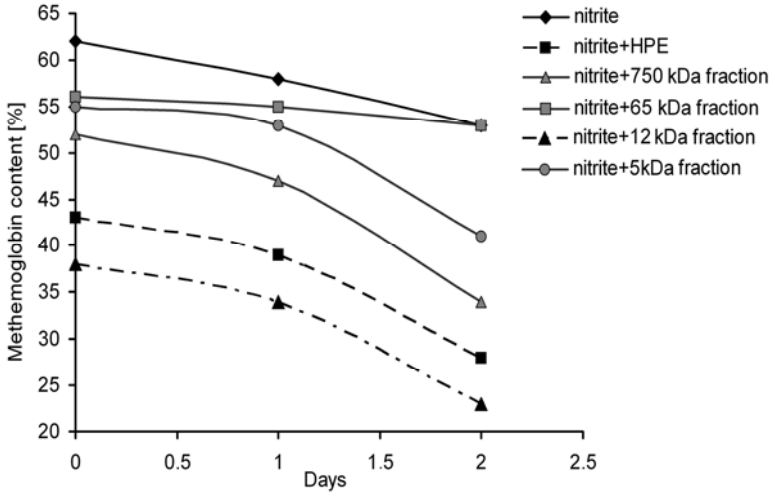

Fig. 5. Methemoglobin reduction in RBC after washing out of nitrite containing solution.

However, addition of HPE and its fractions did not improve low-pH stability of erythrocytes, which is known to be explained by interactions of membrane proteins [14]. The time of 50% low-pH hemolysis decreased after cell incubation with nitrite. Thus HPE does not provide any stabilizing action on membrane structures providing low-pH membrane stability of erythrocytes.

The methemoglobin reducing system is known to be one of the protective mechanisms in erythrocytes. Methemoglobin-reducing enzymes within the erythrocyte restore functional hemoglobin by reducing iron back to its ferrous state. Thus the competing processes of methemoglobin production and reduction are in equilibrium. The effectiveness of such a system depends on numerous factors [19]. Experimental data have revealed that HPE and the fraction with molecular mass of 12 kDa provide the most invigorating effect on the methemoglobin reducing system (Fig. 5).

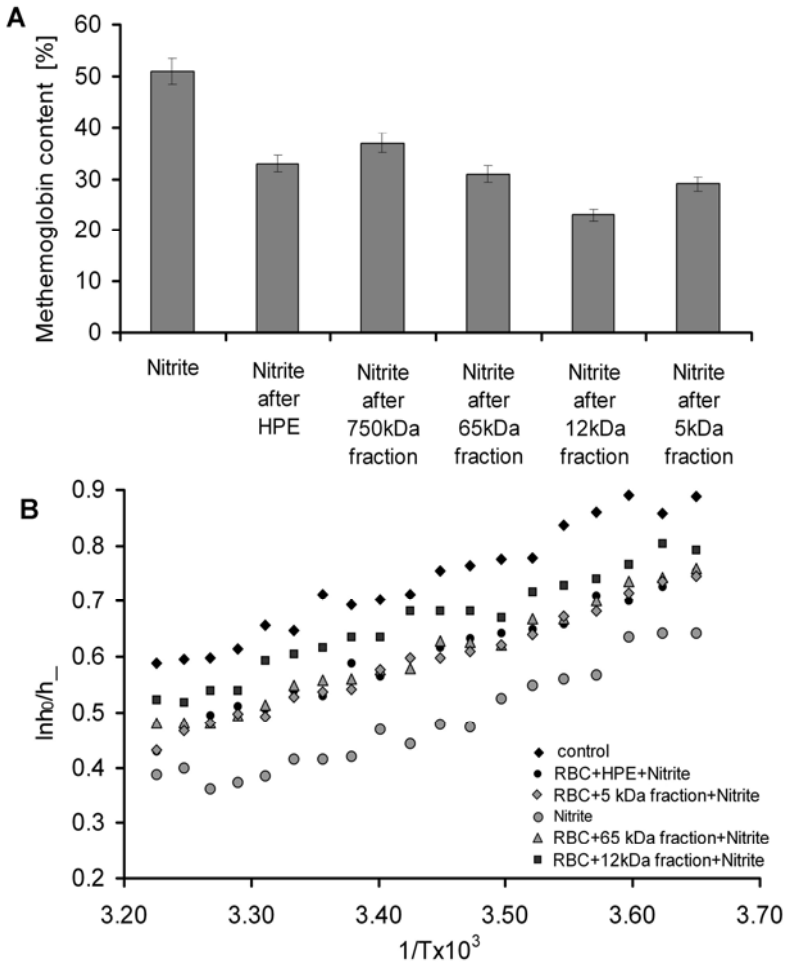

Fig. 6. Influence of preliminary exposure to HPE and its fractions on: A – intracellular methemoglobin content; B – RBC cytosol microviscosity.

In the third experimental group, erythrocytes were preliminarily incubated with HPE or its fractions for 2 h then washed out and incubated with nitrite. The most appreciable lowering of methemoglobin concentration was demonstrated for the fraction with molecular mass of 12 kDa (Fig. 6A). The obtained results match those obtained with the ESR method (Fig. 6B). Preliminary erythrocyte incubation with the extract fraction of 12 kDa allows preservation of the structural-dynamic cytosol state the closest to the control. The protective effect of this fraction may be explained by the presence in HPE of the antioxidant protein thioredoxin with molecular mass of 12 kDa. Thioredoxin is known to be implicated in S-nitrosation (also called S-nitrosylation) activities [20] and to play a significant role in antioxidant protection of erythrocytes [21].

Thus we suppose that extract proteins play a significant role in erythrocyte protection against nitrite-induced oxidative stress. Nitrite-induced oxidative stress of erythrocytes appears to be a convenient model for studying the mechanism of HPEs' biological activity conditioned by their antioxidant properties. Intracellular hemoglobin state, cytosol microviscosity, and low-pH stability are sensitive parameters characterizing oxidative stress.

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