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ORIGINAL ARTICLE

Antioxidant properties of extracts derived from placentae of different gestation terms

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Key Words
Erythrocyte; Human placenta extract; Gestation

Abstract

Objective: Human placenta extract (HPE) is known to possess antioxidant activity due to a high concentration of bioactive substances. This fact contributes to the perspective of its application in clinical practice in order to treat oxidation-induced diseases. In this study, a comparative investigation of antioxidant properties of HPEs derived from placentas of the 38 weeks gestation terms and post maturity is provided.

Methods: Antioxidant activity of aqueous-saline HPEs was evaluated spectrophotometrically using ferric reducing power assay, $ABTS^+$ cation radical decolorization assay, chelating ability assay and total phenolics assay. HPE biological activity was evaluated by ability to decrease methemoglobin formation in a model experiment with erythrocytes under nitrite-induced oxidative stress.

Results: Total $ABTS^+$ scavenging and chelating abilities of extracts derived from placenta of 38 weeks gestation term is higher in comparison with full term spontaneous delivery (40 weeks). Ferric reducing power of extracts does not differ regarding gestation term. Treatment of human erythrocytes with HPE of earlier gestation term significantly attenuates a nitrite-induced oxidative damage. However, HPE derived from placenta of 40 weeks gestation (full term) does not possess protective effect against nitrite induced oxidative stress in erythrocytes.

Conclusion: Treatment of human erythrocytes with HPEs of earlier gestation term significantly attenuates a nitrite-induced oxidative damage.

INTRODUCTION

Human placenta and its preparations are known to possess various therapeutic activities [1, 2]. Since reactive oxygen species (ROS) accumulation is considered as a contributing factor in various diseases, therapeutic activity of placenta may be explained by the presence of bioactive substances possessing antioxidant properties like enzymes, phenolic compounds, ascorbic acid, uric acid, some SH-group containing compounds, reduced glutathione as well as protein amino acids. Thus human placenta extracts (HPE) have been shown to scavenge hydroxyl radical, nitric oxide, superoxide radical; to reduce ferric iron; to chelate transition metal ions and to prevent lipid peroxidation [3, 4]. These facts may contribute to HPE application in clinical practice in order to treat various oxidation-induced diseases.

It is widely known that during a normal pregnancy placenta progressively changes either in morphological or physiological senescence [5]. Herewith content and activity of antioxidants in placenta may change as gestation progresses [6, 7]. Thus studying of antioxidant activity of the extracts derived from placenta of different gestation term is of special importance for both general biological and applied values.

This research represents the results of comparative investigations of two placenta gestation terms, *i.e.* 38^{th} and 40^{th} weeks, on antioxidants properties of its extracts.

MATERIALS AND METHODS

All studies have been approved by the bioethics committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Science of Ukraine and have been performed in accordance with the ethical standards laid down in the 1964 'Declaration of Helsinki'.

Test drug

Human placentas weighing between 400-600 g collected after normal pregnancies from healthy parturients (23-28 years old Caucasians) with their informed consent at the time of Cesarean (C)-section at 38 weeks of gestation (n = 10) and full term spontaneous delivery (40 weeks gestation, n = 10) were immediately placed under ice. Subjects were excluded if there was evidence of fetal anomalies, intrauterine growth restriction, diabetes, hypertension, anemia, tobacco or drug use or other medical or obstetric complications.

The amniotic membrane and umbilical cord were removed. Then placentas were minced into small pieces and washed with cold normal saline. Aqueous-saline extracts were obtained from homogenate of placenta by 12 h exposure in physiological saline at 4ºC followed by filtration through a 0.45 μm membrane filter (Millipore Corp, Cork, Ireland). Content of proteins in extracts was measured using spectrophotometric method [8]. Separate fractions of extracts were obtained using gel-chromatography method on 27 x 1 cm column (Sigma) with Sephadex G-100 and G-200 (Loba Feinchemie, Fischamend, Austria). All spectrophotometric measurements were carried out using Pye Unicam SP-8000 (Cambridge, UK).

Chemicals

2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt (ferrozine), Folin-Ciocalteau reagent, gallic acid (3,4,5-trihydroxybenzoic acid) and L-ascorbic acid was purchased from Sigma (Steinheim, Germany); trichloroacetic acid, iron(III) chloride ($FeCl_3$), iron(II) chloride ($FeCl_2$) and sodium carbonate solution (Na_2CO_3) from Merck (Darmstadt, Germany); potassium ferricyanide ($K_3Fe(CN)_6$), ammonium persulfate and sodium nitrite from Sigma (St. Louis, MO, USA); phosphate-buffered saline (PBS) from Medicago (Uppsala, Sweden).

Spectrophotometrical antioxidant activity assays

A complex approach was chosen in order to evaluate non-enzymatic HPE antioxidant capability and estimate contribution of different antioxidant centers.

Ferric reducing power

The capacity to reduce the ferric-ferricyanide complex to the ferrous-ferricyande complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation [9]. For this purpose, 0.2 ml of the experimental sample were mixed with phosphate buffer (0.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (0.5 ml, 1%). The mixture was incubated at 50ºC for 20 min. Aliquots (0.5 ml) of trichloroacetic acid (10%) were added to the mixture. The upper layer of the solution (2.5 ml) was mixed with distilled water (2 ml) and $FeCl_3$ (0.1 ml, 0.1%). The absorbance was measured at 700 nm in a spectrophotometer. L-Ascorbic acid was used as a standard. Increased absorbance of the reaction mixture indicates increased reduction capability.

Fe^{2+} chelating ability

Ferrous ion (Fe^{2+}) chelation by HPE was estimated by the ferrozine assay [10]. HPE (0.2 ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). Then, the mixture was shaken vigorously and left standing at room temperature for 10-15 min. The solution was mixed with distilled water (3 ml) and the absorbance of the mixture was measured spectrophotometrically at 562 nm. The results were expressed as the percentage of inhibition of the ferrozine-Fe^{2+} complex formation.

$ABTS^+$ radical cation decolorization assay

The spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined according to the method of Re *et al* [11]. $ABTS^+$ cation radical was produced by the reaction between 7 mM $ABTS^+$ in H_2O and 2.45 mM potassium persulfate, stored for 12 h in the darkness at room temperature. Before usage, the $ABTS^+$ solution (0.1 ml) was diluted to get an absorbance of 0.7 ± 0.025 at 734 nm with distilled water. Experimental sample was added to the obtained solution and the kinetics of $ABTS^+$ radical decolorization was recorded. Results were presented as percentage changes in absorbance of initial $ABTS^+$ solution.

Total phenolic compounds assay

Total phenolic content was evaluated by Folin-Ciocalteau method as described earlier [12]: 0.15 ml of Folin-Ciocalteau reagent in 1.35 ml H_2O was added along with 1.2 ml of 7.5% (w/v) sodium carbonate to 0.1 ml of the sample. Then the absorbance was taken at 725 nm after 40 min of incubation at 22°C. Total phenols were calculated using standard curve of gallic acid. The results were expressed in the form of gallic acid equivalents (GAE).

Blood sample preparation

Human red blood cells (RBC) were obtained from the whole donor blood by tree times centrifuging at 1500g at 4ºC for 10 min and washing out with physiological solution. Then erythrocytes were diluted in concentration 1:1 ratio with 5 mM phosphate-buffered saline (pH 7.4). Methemoglobin content in erythrocytes differs within the limits from 0.8 to 1.2%. During the experiments, erythromass was associated with the equal volume of HPE or its separate fractions.

In order to investigate nitrite influence on erythrocyte and to find out if HPEs possess any protective effect against nitrite-induced oxidation the samples were divided into three parts: ***(1)*** the first one contains erythrocytes in PBS; ***(2)*** the second one was erythrocytes after incubation for 20 min with nitrite solution, which did not lead to the erythrocyte hemolysis but to partial oxidation of intracellular hemoglobin; and ***(3)*** the last one was erythrocytes after simultaneous incubation for 20 min with sodium nitrite and HPEs or its separate fractions. Sodium nitrite was dissolved in 5 mM phosphate buffer saline.

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All experimental samples were then washed out of the nitrite-containing medium and methemoglobin content in cells was evaluated spectrophotometrically [13].

Statistical analysis

Statistical analysis was carried out using Statgraphics Plus version 2.1 (Manugistic; Rockville, MD, USA). All the analyses on antioxidant activity for each placenta were done in triplicate set. The data were presented as mean ± standard deviation. The data were analyzed by *t*-test to find out difference between sample means. Values at $P < 0.05$ were considered significant.

RESULTS

Protein concentration assay

Protein concentration in HPEs derived from placentae of 38 weeks of gestation was statistically lower than in case of placentae delivered at full term (8.75 ± 2.61 and 23.13 ± 5.97 mg/ml, correspondingly).

Total phenolic compounds assay

Phenolic compounds, in particular tryptophan, phenylalanine, vitamin E, ubichinons may contribute directly to antioxidative action by scavenging free radicals [14, 15]. Values of the total phenolic content for extracts derived from placentas at 38 and 40 weeks' gestation term were equivalent to 0.46 ± 0.11 and to 1.24 ± 0.46 mM of gallic acid correspondingly.

Ferric reducing power

Reducing power appears to be one of the antioxidant characteristics of bioactive substances. This ability may be connected with the presence of antioxidants able to be donors of electrons. One of the methods for reducing activity assessment is based on analyzing substance capacity to reduce ferric to ferrous ion. Antioxidants which are estimated by this method are known to be mainly substances of low-molecular masses (such as uric acid, ascorbic acid, phenolic compounds, *etc*) and are known to possess a high redox potential [16]. There was no significant difference between reducing power values for placenta of different gestation terms. Values of ferric reducing power for HPEs derived from placenta of 38 weeks gestation and full term were equivalent to 1.12 ± 0.51 and to 1.11 ± 1.54 M of ascorbate correspondingly.

Chelating ability

One of the main antioxidant mechanisms of biological macromolecule protection in extracellular medium is provided by chelating compounds, binding transition metal ions and preventing their participation in peroxide decomposition reactions (Fenton type reactions) with production of hydroxyl radicals [17].

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased. The experimental data has revealed the dramatic changes in chelating ability of HPEs depending on gestation term. Thus chelating ability of extracts derived from different placentae at 38 weeks gestation makes up 13.42 ± 3.79% of bound iron. Extracts derived from placentae at 40 weeks gestation (full term) as well as their fractions do not possess such activity.

$ABTS^+$ radical reducing activity

The ABTS cation radical is known to react with most antioxidants including thiols, ascorbic acid, uric acid and phenolics. During this reaction, the blue $ABTS^+$ radical is converted back to its colorless neutral form [18, 19]. The profiles obtained employing kinetics of $ABTS^+$ decolorization by extracts derived from preterm placenta indicated the occurrence of fast decay (reaction time less than 10 sec), and the slow one was observed after a longer time period (Fig.1[2]).

The data obtained for full gestation term placenta extracts present an overshooting, with a minimum in $ABTS^+$ concentration at 10 sec, followed by a partial recovery of the radical absorbance (Fig.1[3-4]). The observed overshooting may be explained with the occurrence of reversible cross-combination reactions (Fig.1[3]).

Taking into account that in all cases the kinetics of $ABTS^+$ radical reduction included 2 phases, activity of both rapid and slow scavenging antioxidants were assessed. Activity of antioxidants responsible for rapid scavenging was evaluated by decolorization during first 10 sec; activity of antioxidants responsible for slow scavenging was estimated by decay during the next 390 sec.

The obtained values of total $ABTS^+$ reducing activity differ in accordance to placenta gestation term. Such an activity is significantly higher in the case of 38 weeks and it is due to activity of slow scavenging centers of extract (Table 1).

Table 1. $ABTS^+$ radical scavenging activity of extracts derived from placenta of different gestation terms (%).

Gestation term	38 weeks	Full term
Rapid scavenging activity	37.02 ± 11.62	28.76 ± 4.08
Slow scavenging activity	25.16 ± 5.15	8.61 ± 11.12*****
Total antiradical activity (400 sec)	62.43 ± 14.08	37.37 ± 11.89*****

*P < 0.05 in comparison with 38 weeks gestation

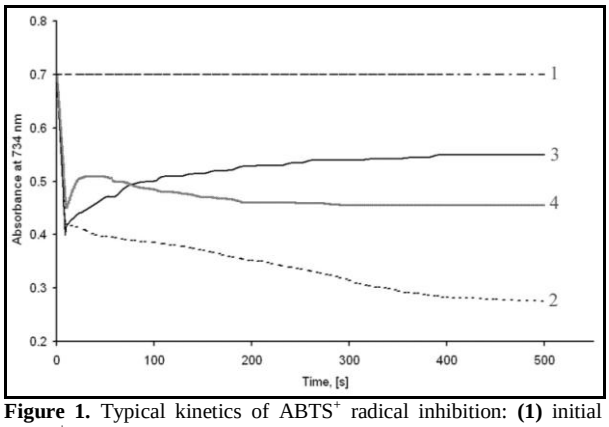

Figure 1. Typical kinetics of $ABTS^+$ radical inhibition: **(1)** initial $ABTS^+$ radical solution; **(2)** HPE derived from 38 weeks gestation placentae; **(3-4)** HPE derived from full term placentae.

Decolorization assay of separate fractions has revealed that almost all fractions derived from HPEs of 38 weeks' gestation term possess $ABTS^+$ radical scavenging activity and contain both rapid and slow scavenging centers. The lowest levels for both rapid and slow scavenging were observed in fractions of high molecular masses (about 750 kDa).

The obtained results for extracts derived from placentas at full term gestation match with these for their fractions. The oxidation phase was observed for HPEs' separate fractions of about 40, 30 and 12 kDa.

Nitrite-induced oxidative stress in erythrocytes

Different biological models are widely used in order to evaluate efficacy of novel antioxidants and mechanisms of their action. One of such a model appears to be erythrocytes under nitrite-induced oxidative stress. Nitrite is known to be able to penetrate erythrocyte membrane and, in case of high extracellular concentrations, to oxidize hemoglobin [20]. In the present work extracts with no hemolytic activity were used.

Our previously reported data revealed that simultaneous incubation with nitrite and HPE of 38 weeks gestation or its separate fractions allows lowering erythrocyte methemoglobin concentration. We also found the fraction with molecular mass of 12 kDa to be the most effective [21]. Extracts derived from placentas at 40 weeks full term gestation as well as their separate fractions do not possess any protective effect against nitrite-induced oxidative stress (Table 2).

DISCUSSION

One of the key mechanisms of various pathological states of organisms appears to be free radical accumulation due to imbalance between antioxidant and pro-oxidant systems [22]. This fact as well as restriction in synthetic antioxidants application, because of their toxic effect, contributes for raising an interest towards new sources of natural antioxidants which may be supplied to the organism as special pharmaceuticals. Placenta extracts appear to be one of the perspective natural antioxidant sources. At least two kinds of antioxidants are active in these biological systems: ***(1)*** enzymatic antioxidants like superoxide dismutase, catalase and various peroxidases; and ***(2)*** nonenzymatic antioxidants like chelating proteins (ferritin, ceruloplasmin, *etc*), thiols (glutathione, thioredoxin, *etc*), amino acids, small molecules (phenols, polyphenols, uric acid, vitamins, *etc*), certain hormones like estrogen and melatonin [3, 4, 23].

Chelating ability may be connected with specific proteins, methionine residues as well as uric acid presence [17, 24, 25]. Slow scavenging of $ABTS^+$ radical by HPEs is presumably conditioned by amino groups of proteins able to quench free radicals. The protective effect of 12 kDa fraction at 38 gestation weeks towards erythrocytes may be explained by the presence of the antioxidant protein thioredoxin. Thioredoxin is known to play a significant role in antioxidant protection of erythrocytes [26]. Absence of such an activity in case of full term placentae matches with decreasing of slow reducing $ABTS^+$ radical centers activity due to oxidative effect in 12 kDa fraction.

With regard to the role of extract proteins in erythrocyte protection against nitrite-induced oxidative stress; since parameters characterizing antioxidant activity of non-protein substances (ferric reducing power and rapid scavenging of activity $ABTS^+$ radical) do not significantly change regarding gestation term the observed alterations are assumed to be connected with proteins. As protein content was lower in HPEs derived from placenta of 38 weeks gestation term in comparison with the full term placentas, one the difference in antioxidant activity may be probably caused by the loose of protein activity.

Previously reported data have revealed that throughout gestation the metabolic activity of placental mitochondria rises leading to increasing of reactive ROS production, mainly the superoxide anion [27, 28]. The placenta also produces other ROS, including nitric oxide, carbon monoxide and peroxynitrite which have pronounced effects on placental function such as trophoblast proliferation and differentiation, and vascular reactivity [23]. Thus, the nitric oxide radical (NO•) plays an important role in the regulation of fetal-placental blood flows, in maintaining low-basal tone and attenuating the vasoconstrictive effects of thromboxane and endothelin [29]. However, the interaction of nitric oxide and superoxide anion yields the peroxynitrite anion ($ONOO^-$), a powerful oxidant which is known to cause lipid peroxidation, lead to covalent modification of proteins by nitration of tyrosine residues, and oxidize sulfhydryl groups; hence

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

Gestation term	Control	Physiological solution	HPE	750 kDa	65 kDa	12 kDa	5 kDa
38 weeks	2 ± 0.48	55.1 ± 3.5	38 ± 4.1*****	44 ± 3.48*****	45 ± 3.81*****	34 ± 4.12*****	51 ± 3.9
Full term	2 ± 0.48	53.2 ± 4.16	54.8 ± 4.4	57 ± 4.51	55 ± 5.23	56 ± 3.92	55 ± 3.86

*****P < 0.05 compared with physiological solution

altering their activity and properties. It should be pointed out that vitamins C and E have little influence on the development of peroxynitrite in contrast to melatonin which is peroxynitrite scavenger [29-31].

The oxidant-antioxidant balance varies as the gestational week increases and the oxidation processes are increased in pregnancy [32, 33]. The increase in free radicals becomes especially profound in the late period of pregnancy and leads to enhancing antioxidant mechanisms to compensate for the raised oxidative stress [34, 35]. Hence we speculate about free radical accumulation in placenta of full gestation term and, as a result, loosing of antioxidant activity of HPEs. Higher content of proteins and phenolic compounds in HPE derived from placenta of full term may be an evidence of better extraction but not of higher antioxidant properties of extracts.

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