Acta Veterinaria Hungarica 49 (4), pp. 413–419 (2001)

ERYTHROCYTE OSMOTIC FRAGILITY TEST AS THE MEASURE OF DEFENCE AGAINST FREE RADICALS IN RABBITS OF DIFFERENT AGE

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(Received May 7, 2001; accepted September 5, 2001)

Peroxidation of the unsaturated bonds of membrane lipids increases fragility and cellular lysis of red blood cells. Erythrocyte susceptibility to the free radicals (peroxyl radicals) generated *in vitro* by 2,2'-azo-bis(2-amidinopropane) hydrochloride (AAPH) was evaluated and expressed as 50% maximal haemolysis time (HT_{50}) in 3 groups of rabbits of different age. Erythrocytes of 1.5-month-old rabbits were more sensitive to free radicals than those of 3.5- and 6-month-old ones. In the three groups, significant negative correlation (r = –0.8 to –0.98) between the lipid peroxidation rate (thiobarbituric acid reactive substances; TBARS concentration) in blood plasma and the erythrocyte resistance to free radicals was found. This result suggests that the plasma antioxidant defence system is interrelated with that of the red blood cells and that the erythrocytes can be a good model for studies of oxidative stress. The simple haemolysis test reflecting the free radical defence can be useful for evaluating the antioxidant properties of various compounds.

Key words: Erythrocyte, free radicals, oxidative stress, lipid peroxidation

Oxidative stress results from an imbalance between the effect of pro-oxidants and the antioxidant defence mechanisms of the body. Mammalian red blood cells (RBC) are particularly susceptible to oxidative damage because: *(1)* being oxygen carrier, they are exposed uninterruptedly to high oxygen tension, *(2)* they have no capacity to repair their damaged components, and *(3)* their membrane components are susceptible to lipid peroxidation (LPO) (Galleano and Puntarulo, 1995). The oxidative modification of the membrane increases fragility of the RBC (Stern, 1986; Wagner et al., 1988). Under normal conditions, RBC rich in catalase, superoxide dismutase, glutathione, glutathione reductase, glutathione peroxidase and a proteolytic system that can hydrolyse oxidatively-modified proteins (Halliwell and Gutteridge, 1989), are capable of preventing most of the adverse effects of oxidative stress (Gutteridge, 1994). Vitamin E (α-

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tocopherol), an extremely effective antioxidant when incorporated into the membrane (Gutteridge, 1978), and melatonin when taken up into human erythrocytes under oxidative stress (Tesoriere et al., 1999), strongly enhance RBC resistance to oxidative lysis. These lytic processes can be evaluated by measuring the free radical-induced RBC haemolysis and utilised to assess the antioxidant properties of natural compounds or drugs including urate, ascorbate, tocopherol, butylated hydroxytoluene and probucol (Blache et al., 1991). One of the advantages of this haemolysis test is the possibility to monitor *ex vivo* the time course effects of a supplementation with various antioxidants (Velasquez-Pereira et al., 1998).

Plasma transferrin and lactoferrin themselves act as antioxidants by binding iron ions (Gutteridge et al., 1981). The plasma copper-containing protein ceruloplasmin plays an essential role in the process. It has a ferrooxidase-activity and oxidases ferrous ions to the less reactive ferric state while reducing oxygen to water, thereby inhibiting iron-dependent LPO (Gutteridge et al., 1980). The ceruloplasmin-catalysed oxidation of ferrous ions does not release any damaging oxygen radicals, as they are kept on the active site of the protein (Gutteridge and Stocks, 1981). Plasma albumin binds copper ions and inhibits copper ion-dependent LPO and hydroxyl radical formation (Gutteridge, 1986). Copper ions can attach SH-groups on the membranes of erythrocytes and facilitate their oxidative damage.

Several methods currently exist for estimating the LPO (for review see Halliwell and Grootveld, 1987). Based on the reaction of malondialdehyde (MDA), a breakdown product of lipid peroxides, with thiobarbituric acid, the measurement of thiobarbituric acid reactive substances (TBARS) has been commonly used to check the products of LPO (Hessler et al., 1983). Although some reports suggest that MDA is an artefact of the TBARS procedure, in most of the biological systems it is a genuine product of LPO (Slater and Cheeseman, 1988).

In the present study erythrocyte susceptibility to oxidative stress was evaluated *in vitro* by submitting cells to an organic free radical generator. To examine the relationship between erythrocyte osmotic fragility and plasma antioxidant defence, the erythrocyte resistance against free radicals and the LPO end-product malondialdehyde were determined in plasma by the thiobarbituric-acid assay.

Materials and methods

Animals

The experiments were performed on White New Zealand rabbits aged 1.5, 3 and 6 months (n = 30) and bred in our laboratory. The animals were kept in separate metal cages at a room temperature around 20 °C and exposed to a 10:14 light-dark cycle. They were provided with commercial pellets for rabbits and drinking water *ad libitum*. Blood was collected with heparin to provide erythrocytes and plasma, placed on ice and transported to the laboratory.

Reagents

1,1,3,3-Tetraethoxypropane (MDA, an external standard), trichloroacetic acid, thiobarbituric acid and n-butanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) from Polysciences (Warrington, PA, USA).

Erythrocyte susceptibility to free radicals

Susceptibility of erythrocytes to free radicals generated *in vitro* was evaluated as described by Girodon et al. (1997). Erythrocytes were isolated by centrifugation of heparinised blood for 10 min at 2000 × *g* at 4 °C, then the plasma was pipetted out, the RBCs were washed three times with 0.15 M NaCl and resuspended at 15% haematocrit with 0.15 M NaCl. Washed erythrocyte suspensions were exposed to peroxyl radical generated by decomposition of 0.15 M AAPH at 37 °C for 4 to 4.5 h. At 30-min intervals, 0.02 ml of the incubation mixture was diluted 200 times with 0.15 M NaCl and centrifuged at 9000 × *g* for 1 min. The released haemoglobin content of the supernatant was evaluated by measuring the absorbance at 405 nm against 0.15 M NaCl. Results were expressed as 50% of maximal haemolysis time (HT_{50} in minutes), using the sigmoidal Boltzmann curve plotted and fitted for each animal by GraphPad Prism computer programme for nonlinear regression.

Assay of thiobarbituric acid reactive substances (TBARS) in plasma

The amount of TBARS in plasma was determined by the method of Ledwożyw et al. (1986) and expressed in MDA. In short, 0.25 ml sample was mixed with 1.25 ml of 1.22 M trichloroacetic acid in 0.6 M HCl and allowed to stand for 15 min. To this mixture 0.75 ml of thiobarbituric acid solution was added (obtained by dissolving 500 mg thiobarbituric acid in 6 ml of 1 M NaOH then adding 69 ml H_2O) and heated for 30 min at 100 °C in an electric heater. After cooling to room temperature 2 ml of n-butanol was added, the mixture was shaken vigorously for 3 min and centrifuged for 20 min at 2000 × *g*. The organic layer, containing the lipid peroxidation end-products, was removed and its absorbance was measured at 532 nm against blank. The results were expressed as nmoles of MDA/1 ml of plasma using 1,1,3,3-tetramethoxypropane as standard. Standard curve was made of MDA solution in concentrations ranging from 2.5 to 50.0 μM.

Statistical analysis

The data were analysed for differences by Student's *t*-test. A difference was considered significant at $P < 0.05$.

Results

The erythrocytes of 1.5-month-old rabbits were more sensitive to free radical damage than those of 3.5- and 6-month-old ones (Fig. 1). HT_{50} values for these three groups of age were 133.0 ± 2.42 min, 144.0 ± 2.49 min and 186.0 ± 7.5 min, respectively ($P < 0.01–0.001$). TBARS content in plasma, expressed as the lipid peroxidation end-product MDA concentration, decreased significantly ($P < 0.001$) with advancing rabbits age from 10.2 ± 0.19 in 1.5-month-old to 6.2 ± 0.31 nmol/1 ml in 6-month-old animals.

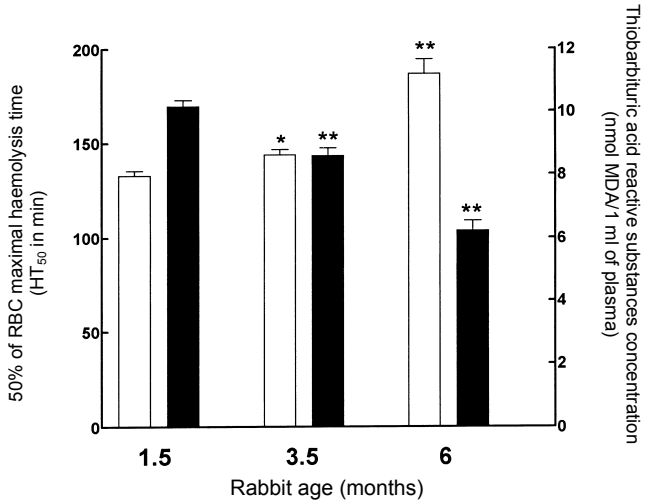

Fig. 1. The HT_{50} values (open bars) and TBARS concentrations (black bars) of the different age groups of rabbits (n = 10 in each group). Mean values ± SEM; $^{*}P < 0.01$; $^{**}P < 0.001$

Significant ($P < 0.01–0.001$) negative correlations between the rate of lipid peroxidation in the plasma and erythrocyte resistance to free radicals were found in all groups of animals (r = from –0.80 to –0.98; Fig. 2).

Discussion

The free radical generator (AAPH) used in the present studies was an azo-compound which thermally decomposed and gave carbon-centred peroxyl radicals and nitrogen. In the presence of air oxygen, peroxyl radicals are produced at a constant rate (Tereo and Niki, 1986). The radicals can abstract hydrogen and peroxidise a lipid substrate, namely linoleic acid and linoleate ester. The utility of this procedure was proven to be useful to assess total antioxidative status as it is not only sensitive to membrane vitamin E and glutathione depletion but also to inhibi-

tion of catalase or glutathione peroxidase (Blache et al., 1991; Girodon et al., 1997). In other tests which have been developed for the measurement of the antioxidant activity of biological fluids, procedures vary as to the free radical generator utilised and to the reproducibility of the generation process. One of the employed procedures is the total radical-trapping potential (TRAP) method developed by Wayner et al. (1985). It uses AAPH as the free radical generator, but this method requires specific oxygen electrode and has limited physiological relevance of AAPH-derived peroxyl radicals (Halliwell and Gutteridge, 1990). Methods devoted to erythrocyte susceptibility to oxidative stress conducted with H_2O_2 or other generators in the presence of a catalase inhibitor measure the rate of lipoperoxidation, the cell deformability or the amount of haemoglobin released (Girodon et al., 1997). The AAPH generator is superior to H_2O_2 because it does not need catalase inhibitor (such as azide) and because peroxyl radicals are produced at a known and constant rate (Yamamoto et al., 1986) which results in a low variability (Girodon et al., 1997).

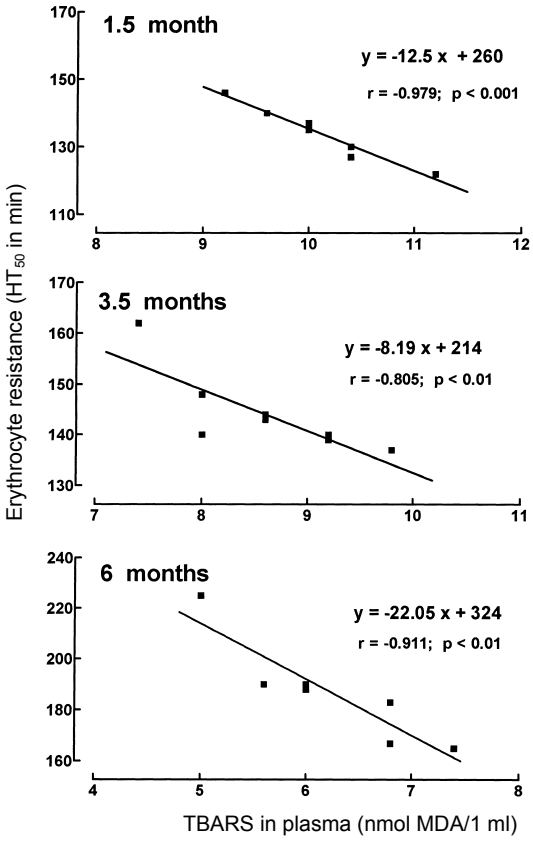

Fig. 2. Correlations between TBARS concentration of the blood plasma and the resistance of erythrocytes to free radicals

The present study shows that the RBC susceptibility to free radicals is influenced by the age of the animals (Fig. 1). The erythrocytes of young rabbits (1.5 months old) were less resistant to free radical damage than those of older animals. Using the free radical-induced haemolysis test, Girodon et al. (1997) compared the oxidative damage in young (42 years old) and elderly (> 65 years) humans. In elderly subjects, the RBC antioxidant defence was reduced and could be improved with antioxidants (vitamins C and E, β-carotene) supplementation. These antioxidants were also beneficial in improving the erythrocyte osmotic fragility in rats (Kraus et al., 1997*a,b*).

As shown in Fig. 2, a significant correlation between erythrocyte susceptibility to free radicals and the rate of LPO (TBARS level) in plasma exists within each group of age. The TBARS assay, as an index of free radical degradation of polyunsaturated fatty acids measures two main products: malondialdehyde and 4-hydroxynonenal (Reiter, 1995). This aldehyde can cause cross-linking of lipids, proteins and nucleic acid (Freeman and Crapo, 1982; Flohe et al., 1985).

Our results confirm the suggestion of Stocker and Frei (1991) that plasma antioxidant defence is interrelated with that of the RBC, as illustrated by variation in RBC HT_{50} against the plasma TBARS level. The HT_{50} is influenced more by chain-breaking antioxidants than by iron-oxidising or iron-binding antioxidants in plasma (Brzezińska-Ślebodzińska, unpublished data). This result suggests that the plasma chain-breaking antioxidants may reflect the amount of the same antioxidants of the erythrocyte membrane, which most likely provides primary protection against haemolysis induced by peroxyl radicals.

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