

Relationship Between Aging and Susceptibility of Erythrocytes to Oxidative Damage: In View of Nutraceutical Interventions

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ABSTRACT

Twelve (12) healthy elderly subjects were divided into two groups: (a) those given an antioxidant/NO-modulating fermented papaya preparation (FPP) 9 g/day for 4 weeks, and (b) a placebo group. No protein/lipid distribution in erythrocytes (RBC) membranes was noted among different ages and treatments. Higher RBC concentration of malondialdehyde and nitric oxide synthase were found in the elderly ($p < 0.05$ versus "young" controls), whereas superoxide dismutase was unaltered. Such abnormalities were prevented by FPP supplementation ($p < 0.01$). RBC and RBC ghosts showed an enhanced susceptibility to lipid peroxidation by using cumene hydroperoxide ($p < 0.01$ versus young) but FPP supplementation significantly protected intact RBC ($p < 0.05$). These preliminary data suggest that nutraceuticals with antioxidant/NO-regulating properties significantly protect from RBC oxidative damage, and are potential weapons for the aging process and chronic and degenerative diseases.

INTRODUCTION

ERYTHROCYTES AND ERYTHROCYTE MEMBRANES are a feasible biological system to study in aging-related investigations, because unsaturated lipids in the cell membrane, amino acids, and DNA nucleotides represent specific target for free radical damage.^{1,2} Moreover, recent studies point out the role of oxidative damage to biomembranes in a number of chronic inflammatory and degenerative diseases. Indeed, despite the fact that no overt changes of membrane components have been reported in erythrocytes (RBC) with advancing age,³ peroxinitrite anion-related damages to platelets and RBC have been implicated in age-related neu-

rodegenerative disease. Although there are still some conflicting results,⁴ it appears that erythrocytes from elderly individuals and aging animals are highly susceptible to oxidative stress.⁵⁻⁷ Although these derangements may represent an epiphenomenon of more complex epigenetic abnormalities, a tentative therapeutic intervention on the expected higher RBC vulnerability to oxidative stress might be of interest. Thus, given that susceptibility of erythrocytes to oxidative damage is altered during the aging process, the authors' aim was to assess whether this phenomenon could be beneficially influenced by a specific nutritional supply. Thus, a functional food which has been shown in controlled experimental and clinical

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studies to possess potent antioxidant/NO-modulating properties was used.⁸⁻¹⁰ In particular, the authors have recently shown in alcoholic liver disease patients that this compound could significantly improve blood hemorrheology as a whole and RBC membrane fluidity.¹¹ Moreover, preliminary data from Rachmilewitz and Fibach seem to suggest that such nutraceutical could decrease the intracellular content of reactive oxygen species (ROS) and concomitantly increase the glutathione levels in RBC of patients with thalassemia intermedia.¹² Cumene hydroperoxide (CumOOH) was used as an oxidative stress test of erythrocyte from aged people. Its lipophilicity makes it a feasible trigger of peroxidative cleavage of membrane lipids and proteins alterations in erythrocytes.¹³

MATERIALS AND METHODS

Subjects

The authors' study group consisted of 12 non-smoking healthy elderly patients (mean age: 68, range 62-75). Major invalidating diseases, such as prior or ongoing cancer, dyslipidemia, chronic illness requiring steroids or immunosuppressive agents, allopurinol treatment, chronic renal failure and cardiorespiratory diseases, were regarded as exclusion criteria. Subjects were randomly divided in two groups matched for age and dietary habits, which were allocated to a 4-week treatment period. One group was given a GMP-, ISO9001/14000- certified fermented papaya preparation (FPP, Osato Research Institute,

Gifu, Japan) 9 g/day by mouth in the morning, 1 hour after breakfast and fasting for further 30 minutes afterward, whereas the control group received the same amount of placebo (flavored sugar devoid of any antioxidant property). As an age-control group, a group of 8 young (mean age: 31, range 22-34) healthy subjects were considered as well. All subjects had normal routine blood chemistry and were instructed not to take aspirin or NSAIDs for at least 3 weeks before blood sampling.

Blood antioxidant status

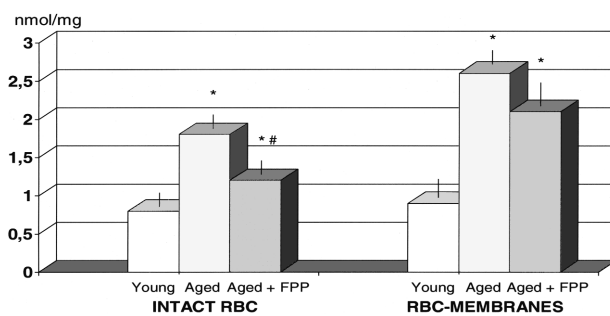
The following parameters were measured: plasma lipid hydroperoxides by hemoglobin catalyzed oxidation of 10-N-methylcarmoyl-3,7-dimethylamino-10-H-phenothiazine after treatment with phospholipase D. Plasma content of α -tocopherol was measured by high-performance liquid chromatography (HPLC).

Fractionation of erythrocytes by age

Fasting blood samples obtained from subjects in the morning were centrifuged at 1000 g for 10 min and put into heparinized tubes. Erythrocytes were separated from plasma and buffy coat and washed three times with 10 vol of phosphate-buffered saline (PBS, pH 7.4) to obtain erythrocyte sediments. Erythrocytes were then fractionated by Percoll discontinuous density gradient/centrifugation and age-stratified by determining their MCV and glycohemoglobin. A two-layer gradient was obtained with specific density values between 1.100 and 1.124 g/mL. At the end of centrifugation, the cells were recovered at the interface of the Percoll <1.124 g/mL corresponding to the young cells rich in reticulocytes.

Preparation of erythrocytes and their membranes

After removal of the buffy coat the erythrocyte sediment was washed three times with phosphate buffered solution. White erythrocyte membranes were prepared by hypotonic hemolysis using standard procedures with Tris buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonylfluoride and 0.01% butylated hydroxytoluene and membranes were finally suspended in isoosmotic Tris-HCl.



* $p < 0.01$ vs young; # $p < 0.05$ vs aged without FPP

FIG. 1. Effect of CumOOH oxidative stress test on RBC-MDA in intact RBC and RBC membranes: effect of FPP.

Oxidative challenge test

Intact erythrocyte suspension in PBS or membrane (2 mg protein/mL) in 5 mM sodium phosphate, pH 7.4 was used for lipid peroxidation stress test induced by addition of CumOOH (final concentration, 0.2 mM). After 60 min, cell suspensions for intact erythrocytes were centrifuged at 7000 g for 1 min to sediment the erythrocytes. The pellet was washed with PBS and white membranes were prepared. The membrane pellets were washed twice with 15 vol of 10 mM Tris buffer (pH 7.4). The pellets were resuspended in 1.0 mL with same buffer. All membrane suspensions were used for protein analysis. Parameters tested were: MDA by HPLC with acetonitrile/30 mM TRIS buffer, pH 7.4 as the eluent, and SOD activity by measuring the nitroblue tetrazolium reduction by O₂ generated by the xanthine/xanthine-oxidase system.

Erythrocyte lipid and protein analysis

Total lipid extraction was performed in order to determine phospholipids and cholesterol and their composition by densitometric analysis. RBC ghost protein also was analyzed by polyacrylamide gel electrophoresis; they were stained with Coomassie blue and their densitometric peaks were weighed to determine the relative amounts.

RESULTS

The plasma concentration of hydroperoxide and of α -tocopherol and protein-lipid distribution and phospholipid composition in RBC were comparable among young and elderly subjects. This applied also when performing the CumOOH test although this triggered in both groups the partial degradation of bands 1, 2, and 3 with formations of high molecular weight polymers (HMWP). SOD level was comparable in both age-groups and was not affected by FPP administration. NOs concentration in RBC and MDA concentration in both RBC and RBC membrane were higher in elderly subjects ($p < 0.05$) and such difference was further enhanced by CumOOH tests (elderly RBC > young RBC, $p < 0.05$). These parameters were found to re-

turn within normal limits in FPP-supplemented group in resting tests, whereas FPP-supplementation significantly decreased RBC but not RBC-membrane susceptibility in elderly subjects ($p < 0.05$) (Fig. 1).

DISCUSSION

Circulating erythrocytes are exposed to high oxygen tension and they also abound in iron, which is a transitional metal promoting the formation of oxygen free radicals. A number of studies have shown that the exposure of erythrocyte membranes are exposed to lipid peroxidation can cause structural abnormalities in proteins and lipids through crosslinking, fragmentation phenomena, and protein-lipid adducts formation.¹⁴ Moreover, the formation of HMWP protein aggregates, as occurred also in the present study, might be independent of lipid peroxidation, as they result from a direct attack of radicals on the proteins, whereas mature RBCs are known to have limited capacity to replace damaged protein by *de novo* synthesis. As reported by others,³ the present authors did not find any gross changes either in the lipid composition or the protein content. However, earlier¹⁵ and quite recent studies suggest that RBC from elderly patients may undergo several oxidative stress-related alterations such as of protein structure and of RBC-membrane enzyme activity.¹⁶ Indeed, in the present study, prior to CumOOH-test RBC from elderly people showed a significantly higher concentration of MDA and NOs, the former also at RBC-membrane level. Such differences were further enhanced under oxidative stimulus, pointing out that RBC from elderly subjects displays a higher susceptibility to oxidative stress. Interestingly, FPP-supplementation enabled such parameters to return within normal "young" limits in intact RBC but not RBC membranes. Different age-related phospholipid-cholesterol molar arrangements altered membrane lipid exposure on the outer surface and lipid asymmetry might be factors to be advocated for to explain such result. Taken altogether, these data might be of interest when considering that higher concentrations of MDA and NOs have been quite recently demonstrated in erythrocytes and platelets of Alzheimer's dis-

ease patients¹⁷ as well as decreased RBC uptake of vitamin E in diabetics¹⁸ and possible links between RBC-oxidative damage and microcirculatory disturbances in middle-aged healthy subjects.¹⁹ Moreover, very recently Rachmilewitz, reviewing in fine detail the issue of oxidative damage in thalassemia, suggested the strong potential of antioxidant therapy.²⁰ Although ROS generated at different sites (i.e., external or internal to the RBC) might have different patterns of effect, thus modifying the directionality of pathologic oxidant stress, the present preliminary data suggests that a nutraceutical intervention might prove to be a useful complementary tool in therapeutic strategies of aging and age-related diseases.

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