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Antioxidant enzymes and fatty acid status in erythrocytes of Down syndrome patients

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The excess of genetic information in patients with Down syndrome (DS) produces an increase in the catalytic activity of superoxide dismutase (SOD1), an antioxidant enzyme coded on chromosome 21. It has been suggested that an increase in oxidative stress in DS patients may cause adverse effects in the cell membranes through the oxidation of polyunsaturated fatty acids (PUFAs). The aim of this study was to evaluate the cellular antioxidant system by determining the catalytic activity of the SOD1, glutathione peroxidase (GPx), catalase (CAT), and glutathione reductase (GR) enzymes and the concentrations of α-tocopherol in red blood cells (RBCs) in a group of 72 DS patients. The profile of fatty acids in the phospholipids of RBC membranes was also evaluated. The activity of the erythrocyte antioxidant enzymes is significantly higher in the DS group than in the control group (SOD1, 635 ± 70 U/g Hb vs 476 ± 67 U/g Hb; CAT, 1843 ± 250 U/g Hb vs 1482 ± 250 U/g Hb; GPx, 23.2 ± 5.3 U/g Hb vs 21.5 ± 3.6 U/g Hb; and GR, 9.32 ± 1.4 U/g Hb vs 6.9 ± 1.3 U/g Hb, respectively). No differences were observed in RBC α-tocopherol concentrations between the two groups studied. Long-chain n6 PUFA (C20:3n6, C20:4n6) concentrations were increased in DS patients, suggesting enhanced Δ-6-desaturase activity. The long-chain n3 PUFA (docosahexenoic acid) does not appear to be affected by increased oxidative stress, probably because of the existence of compensatory antioxidant mechanisms.

Reactive oxygen (RO)[4] species are substances that are released during oxidative metabolism. RO species include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\cdot$) *(1)*. The reactions of RO species with macromolecules can lead to DNA mutations, changes in the structure and function of proteins, and peroxidative damage of cell-membrane lipids *(2)*.

There is a primary defense system against oxidative stress, mediated by sequential enzymatic reactions. In the first step of the process, CuZn-superoxide dismutase (SOD1) catalyzes the dismutation of O_2^- to H_2O_2. Glutathione peroxidase (GPx) and catalase (CAT) then independently convert H_2O_2 to water *(3)*. Any increase in SOD1 catalytic activity, therefore, produces an excess of H_2O_2 that must be efficiently neutralized by either GPx or CAT. Otherwise, H_2O_2 reacts with O_2^-, producing $OH^\cdot$, which is one of the most active RO species. Thus the activity of the first-step (SOD1) and second-step (GPx, CAT) antioxidant enzymes must be balanced to prevent cell damage. In addition to the antioxidant enzymatic system, some micronutrients act as nonenzymatic antioxidants by scavenging RO species. α-Tocopherol (vitamin E) is the most important nonenzymatic antioxidant system in the organism *(4)*. Its action occurs mostly in cell membranes by protecting them from lipoperoxidation. An imbalance between the oxidative stress and the cell antioxidant system may produce cell membrane damage by oxidation of its lipid matrix, particularly polyunsaturated fatty acids (PUFAs), which are the main substrates for lipid peroxidation *(5)*.

Down syndrome (DS) is the most frequently occurring chromosomal disease, consisting of a trisomy of the 21st chromosome. The distal segment of this chromosome

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[4] Nonstandard abbreviations: RO, reactive oxygen; SOD1, CuZn-superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; PUFA, polyunsaturated fatty acid; DS, Down syndrome; RBC, red blood cell; GR, glutathione reductase; and GSH, glutathione.

is the site of the gene encoding the synthesis of SOD. It has been suggested that the excess of genetic information in DS patients is the cause of an increase in the activity of SOD1 in these patients *(6, 7)*.

In recent years, it has been hypothesized that an increase in oxidative stress in patients with DS would account for the appearance of different diseases, such as atherosclerosis, accelerated cell aging, cellular mutagenicity, and the neurologic disorders that often occur in these patients *(8, 9)*.

Some studies have related antioxidant enzyme activity to unspecific lipoperoxidation products such as thiobarbituric acid-reactive substances, including malondialdehyde, in DS *(10, 11)*. Nevertheless, to our knowledge, the relationship between the antioxidant systems and the fatty acid composition of the cell membranes has not been investigated. We have therefore studied the activity of the antioxidant enzymes, the concentration of α-tocopherol, and the fatty acid profile in red blood cells (RBCs) of DS patients to assess their cell protection against RO species.

Materials and Methods

PATIENTS

The activities of erythrocyte antioxidant enzymes and α-tocopherol were studied in 72 patients with DS (mean age, 17.8 ± 15.8 years) and 72 healthy controls (mean age, 14.6 ± 10.8 years). The subjects were divided into 3 age groups: group 1, ages 1–9 years (n = 29); group 2, ages 10–19 years (n = 19); and group 3, ages 20–50 years (n = 24).

Fatty acid profiles of the phospholipids in RBC membranes were determined in 16 DS adult patients (ages 34.4 ± 8.3 years) and in 17 healthy adults (ages 31.4 ± 4.3 years).

We chose to study the fatty acid composition of RBC membranes only in adults because of the difficulties in obtaining blood samples in infants and children, especially those with DS. In fact, adult patients seemed to be the most suitable for the purposes of the study because the oxidative damage may be progressive with time and be particularly evident in adult life. The diagnosis of DS was made on the basis of the phenotype and confirmed cytogenetically.

SAMPLES FOR ANTIOXIDANT ENZYME DETERMINATION

Antioxidant enzyme activities were assayed in blood samples collected with EDTA. Erythrocyte pellets were obtained from 1 mL of fasting venous blood by centrifugation at 500*g* for 10 min at room temperature immediately after the blood was drawn. The plasma and buffy layer were then removed, and the erythrocytes were washed three times in a 9 g/L NaCl solution. Lysed erythrocytes were prepared by putting cells through three freeze-thaw cycles in dry ice and by the addition of five volumes of ice-cold distilled water. Cell membranes were removed by centrifugation, and the supernatant was frozen at −20 °C until the determination of SOD1, GPx, CAT, and glutathione reductase (GR) activities.

The activities of erythrocyte antioxidant enzymes were measured in a Cobas Fara centrifugal analyzer at 37 °C *(12)* and expressed as units of enzyme per g of hemoglobin.

GPx activity was measured by the Plagia and Valentine method *(13)* using *tert*-butyl hydroperoxide as the substrate. Units of enzyme activity were calculated using the millimolar absorptivity for NADPH.

SOD1 activity was assayed by the method of McCord and Fridovich *(14)*. SOD1 activity was expressed as the amount of protein causing a 50% inhibition of cytochrome *c* reduction under the conditions of the assay.

GR activity was determined by the generation of $NADP^+$ from NADPH during the reduction of glutathione (GSH) according to the method of Goldberg and Spooner *(15)*. Units of enzyme activity were calculated using the millimolar absorptivity for NADPH.

CAT peroxidative activity was measured by the reaction of formaldehyde produced from methanol with Purpald to produce a chromophore according to the method of Johansson and Hakan Borg *(16)*. Quantitation was carried out by measuring the absorbance at 540 nm and comparing the results with those obtained with formaldehyde calibrators.

SAMPLES FOR α-TOCOPHEROL DETERMINATION

Fasting blood samples were collected in EDTA tubs. The RBCs were separated within the first 2 h after blood drawing. The blood was centrifuged, and the plasma and buffy layer were removed by aspiration. The RBCs were washed three times with a 9 g/L saline solution containing 5g/L pyrogallol to prevent in vitro α-tocopherol oxidation. After the third washing, the final RBC hematocrit was made up to about 50% and determined for use in calculations. The RBCs were frozen at −80 °C until determinations were made.

RBC α-tocopherol was determined by HPLC in a Perkin–Elmer chromatograph (Perkin-Elmer) *(17)*. We used a 150 × 4.6 mm reversed-phase column packed with 5-μm Nucleosil ODC. The chromatography was carried out using methanol as the eluent at a flow rate of 2 mL/min. The effluent was monitored at 280 nm, and quantitation was made using tocopheryl acetate as the internal standard. The within- and between-day imprecision of the method were 3.5% and 8.4%, respectively, and the detection limit was 10 ng per injection.

FATTY ACID ASSAY

RBC membranes were obtained from 2-mL samples of whole blood. The RBC lipid extraction procedure has been previously described *(18, 19)*. Phospholipids were separated by thin layer chromatography on Silica Gel G-60 (Merck) using the solvent system described by Skipski and Barclay *(18)*. Direct transesterification of fatty acids was immediately carried out in methanol-benzene (4:1, by volume) with acetyl chloride, according to the procedure of Lepage and Roy *(19)*, as has been described previously in detail *(20)*. The benzene extract was evapo-

rated under a stream of nitrogen at 40 °C to complete dryness. The residue was dissolved in 100 μL of benzene, and a 1-μL aliquot was injected into the chromatograph.

Fatty acid methyl esters were quantified by gas-liquid chromatography in a Perkin–Elmer Autosystem chromatograph (Perkin–Elmer) equipped with a flame ionization detector and a 30-m capillary column (0.25-mm i.d.) impregnated with SP-2330 as the stationary phase. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min.

The initial oven temperature was 145 °C. The temperature was then increased at a rate of 4 °C/min to 230 °C, which was maintained for 8 min. The injector port temperature was 250 °C, and the detector temperature was 275 °C. The identification and quantification of fatty acid methyl esters was made possible by the use of an external standard mixture [oleic (C18:1n9), linoleic (C18:2n6), γ-linolenic (C18:3n6), dihomo-γ-linolenic (C20:3n6), arachidonic (C20:4n6), adrenic (C22:4n6), *cis*-4,7,10,13,16-docosapentenoic (C22:5n6), linolenic (C18:3n3), *cis*-5,8,11,14,17-eicosapentaenoic (C20:5n3), *cis*-7,10,13,16,19-docosapentenoic (C22:5n3), and *cis*-4,7,10,13,16,19-docosahexenoic (C22:6n3) methyl esters] supplied by Sigma.

Individual response factors were calculated for each fatty acid. Fatty acids from C18:1n9 to C22:6n3 were measured; unidentified peaks accounted for <0.5% of the total fatty acids. The results are expressed as a percentage distribution of each fatty acid.

CALCULATIONS

The SOD1/GPX+CAT activity ratio was calculated from the logarithms of the activities because of the different orders of magnitude.

The n3/n6 ratio was calculated by dividing Σ n3 PUFAs (C18:3n3, C20:5n3, C22:5n3, and C22:6n3) by Σ n6 PUFAs (C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:4n6, and C22:5n6).

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. The comparison between values obtained in patients and controls was performed by the Student's *t*-test for unpaired data or the corresponding nonparametric Mann–Whitney *U*-test if the conditions of application were not fulfilled. Comparisons among the different age groups were carried out by ANOVA and by the Duncan test a posteriori or by the corresponding Kruskal–Wallis nonparametric test if the conditions of application were not fulfilled. In this event, the Mann–Whitney *U*-test was used for a posteriori comparisons.

ETHICAL CONSIDERATIONS

This work was done in accordance with the current revision of the 1975 Helsinki Declaration and was approved by the ethical committees of the hospitals.

Results

The activities of the RBC antioxidant enzymes were significantly higher in the DS group than in the control group, with an increase in catalytic activity of 37% in SOD1, 8.4% in GPx, 37.4% in GR and 24.3% in CAT (Table 1).

When the data were analyzed according to the age groups studied, we found that SOD1 and GR catalytic activity decrease with age, the activity being significantly higher in the youngest compared with the oldest group (SOD1, group 1 vs group 3; GR, group 1 vs group 3 and group 2 vs group 3) in both the controls and the DS patients (Table 2). In contrast, GPx activity increases with age, the activity being significantly higher in the oldest vs the youngest group (GPx, group 3 vs group 1) in both the controls and the DS patients. No differences were observed in the catalytic activity of RBC CAT among the age groups studied.

No significant differences were observed in the balance of the antioxidant enzyme system as assessed by the SOD1/GPX+CAT ratio in patients with DS compared with the control group (0.59 ± 0.013 vs 0.60 ± 0.015, respectively).

Likewise, no differences were observed in RBC α-tocopherol concentrations between DS patients and controls (4.17 ± 0.76 μmol/L vs 4.24 ± 0.67 μmol/L, respectively).

Table 1. Catalytic activity of the SOD1, GPx, GR, and CAT antioxidant enzymes in RBCs of patients with DS and in controls.

Group	SOD1, U/g Hb	GPx, U/g Hb	GR, U/g Hb	CAT, U/g Hb
Control	476 ± 67	21.5 ± 3.6	6.9 ± 1.3	1482 ± 250
DS	635 ± 70	23.2 ± 5.3	9.3 ± 2.4	1843 ± 250
P	0.0001[a]	0.026[b]	0.0001[b]	0.0001[a]

[a] Student's *t*-test.
[b] Mann-Whitney *U*-test.

Table 2. The influence of age on the catalytic activity of the SOD1, GPx, GR, and CAT antioxidant enzymes in RBCs of patients with DS and in controls (NV).

	Group			
Enzyme activities	1 1–9 Years (n = 29)	2 10–19 Years (n = 19)	3 20–50 Years (n = 24)	*P*[a]
	U/g Hb	U/g Hb	U/g Hb	
SOD1-NV	493 ± 69[b]	487 ± 72	447 ± 52	0.049
SOD1-DS	657 ± 77[b]	643 ± 59	609 ± 59	0.035
GPx-NV	19.5 ± 3.0[c]	21.2 ± 3.2	23.6 ± 2.9	0.020
GPx-DS	21.6 ± 5.3[c]	23.6 ± 2.9	26.7 ± 7.3	0.0001
GR-NV	7.5 ± 1.1[c]	6.8 ± 1.1[b]	5.9 ± 0.8	0.047
GR-DS	11.3 ± 2.0[c]	8.6 ± 1.6[b]	7.5 ± 1.2	0.0001
CAT-NV	1430 ± 227	1435 ± 263	1568 ± 253	NS[d]
CAT-DS	1957 ± 216	1831 ± 254	1808 ± 258	NS

[a] ANOVA or Kruskal-Wallis as required.
[b] $P < 0.05$ vs group 3 (a posteriori test).
[c] $P < 0.01$ vs group 3 (a posteriori test).
[d] NS, not significant.

In addition, RBC α-tocopherol concentrations did not differ between the three age groups studied either in DS patients or in controls: 4.13 ± 0.69 μmol/L vs 4.19 ± 0.72 μmol/L (1–9 years), 4.27 ± 0.74 μmol/L vs 4.31 ± 0.68 μmol/L (10–19 years), and 4.15 ± 0.78 μmol/L vs 4.24 ± 0.63 μmol/L (20–50 years), respectively.

The unsaturated fatty acid concentrations expressed as the percentage of total fatty acids in the RBC membrane phospholipids are shown in Table 3. The C18:2n6 (linoleic acid) was significantly lower in DS patients than in controls. The concentrations of long-chain n6 PUFAs (C20:3n6 and C20:4n6) increased in DS patients compared with the control group. The elongation products of arachidonic acid (C22:4n6 and C22:5n6), however, significantly decreased in DS. Concentrations of long-chain n3 PUFAs were significantly higher in DS patients than in controls (7.17 ± 1.45% vs 6.18 ± 1.14%; $P = 0.041$); increased concentrations of docosahexenoic acid in DS patients accounted for most of this difference. However, there were no differences in the n6 PUFA content between patients and controls (20.56 ± 1.30% vs 20.14 ± 1.60%). As a consequence, the n3/n6 ratio showed a nonsignificant trend to be higher in DS patients than in controls (0.35 ± 0.09% vs 0.31 ± 0.06%).

Discussion

A 37% increase in the catalytic activity of SOD1 and a 24.3% increase in CAT activity was observed in DS patients vs controls. The increase in SOD1 enzyme activity is due to an excess of genetic information *(6, 8, 21, 22)*. It is conceivable that the excess H_2O_2 produced by the action of SOD1 would induce the increase in the catalytic activity of the CAT and GPx.

Table 3. Concentrations (expressed as mean ± SD) of unsaturated fatty acids in the phospholipids of RBC membranes of patients with DS and in controls.

Unsaturated fatty acids	Control, %	DS, %	*P*[a]
C18:1n9	10.4 ± 0.59	11.05 ± 1.13	NS[b]
C10:1n7	0.99 ± 0.12	0.99 ± 0.13	NS
C18:2n6	9.96 ± 1.5	7.68 ± 1.29	<0.0001
C18:3n6	0.09 ± 0.13	0.14 ± 0.09	NS
C20:2n6	0.43 ± 0.14	0.42 ± 0.15	NS
C20:3n6	1.27 ± 0.23	1.64 ± 0.25	<0.0001
C20:4n6	12.12 ± 1.35	13.06 ± 0.95	0.041
C22:4n6	5.60 ± 0.84	4.73 ± 0.43	0.003
C22:5n6	0.60 ± 0.60	0.53 ± 0.10	0.093
C18:3n3	0.085 ± 0.031	0.109 ± 0.04	NS
C20:5n3	0.54 ± 0.17	0.56 ± 0.26	NS
C22:5n3	1.00 ± 0.12	5.56 ± 1.29	NS
C22:6n3	4.62 ± 0.92	5.56 ± 1.29	0.03
Σn6	20.14 ± 1.60	20.56 ± 1.30	NS
Σn3	6.18 ± 1.14	7.17 ± 1.45	0.041
Σn3/Σn6	0.31 ± 0.06	0.35 ± 0.09	NS

[a] Mann-Whitney *U*-test.
[b] NS, not significant.

GPx is an enzyme capable of acting not only on H_2O_2 but also on lipid peroxides. We observed an increase in the GPx catalytic activity (8.4%), although this was lower than that found in other antioxidant enzymes. This increase in GPx activity may be due to an induction of the enzyme by an excess of H_2O_2 and lipid peroxides as an adaptive response to oxidative stress *(21, 22)*. The catalytic activity of GR was also increased in DS patients. To act as an antioxidant, GPx requires reduced GSH, which is then oxidized, losing its activity. The regeneration of GSH is carried out by GR.

The increase in GPx catalytic activity would explain, in part, the increase in the catalytic activity of GR in DS patients. However, in our study GR increases much more than GPx. The activity of GR is related to the concentration of GSH, which acts as more than a substrate for GPx. In fact, GSH functions include not only the scavenging of RO species but also the detoxification of xenobiotics and carcinogens, as well as the regulation of immune functions and maintenance of protein structure, function, and turnover *(23)*. Although it was not measured in our study, low concentrations of GSH might contribute to the increase in GR activity.

Other authors *(11, 24–28)* also report the increase in SOD1 and GPx activities observed in this study. However, in most studies, CAT activity *(24, 27, 28)* was not increased. This may be because we assayed the peroxidative function of CAT to determine enzyme activity, whereas other authors assayed the catalase activity of the enzyme. CAT has a double function; it catalyzes the decomposition of H_2O_2 into oxygen and water (catalase activity) and also oxidizes electron donors such as ethanol, methanol, or phenols (peroxidative activity). In the assay for the peroxidative activity of CAT, formaldehyde produced from methanol reacts with Purpald to produce a chromophore measured at 540 nm, which is compared with the values obtained with formaldehyde calibrators *(16)*. This method is more sensible than the conventional method of determining catalase activity by monitoring the decomposition of H_2O_2 at 240 nm *(29)*. On the other hand, the peroxidative activity is not influenced by the presence of other oxidases in the sample.

Our results indicate that SOD1 activity decreases with age in both the control group and in SD patients. The decrease is of the same degree in both groups and may be due to inactivation of the enzyme caused by the aging process, as has been demonstrated in rats *(30)*. On the other hand, the increase in GPx observed with age may be caused by an increase in the formation of peroxides during the aging process, as occurs in the plasma of DS patients *(31)* and in the brains of rats *(24)*.

An imbalance in the antioxidant enzymes may have adverse effects on cell membranes through the indiscriminate oxidation of susceptible molecules such as PUFAs. Cells need to maintain the balance or ratio between the first- and second-step antioxidant enzymes to prevent the excessive generation of the hydroxyl radical.

Some authors *(28, 32)* have suggested that the alteration in the SOD1/GPx+CAT ratio rather than the absolute concentrations of antioxidant enzymes is an important determinant of cellular damage, because changes in this quotient correlate well with an increase in lipid damage. In our study, the lack of differences in the SOD1/GPx+CAT ratio in DS patients vs controls indicates that the primary increase in SOD1 in DS patients would provoke an adequate adaptive rise in both GPx and CAT as a compensatory mechanism, thus maintaining the equilibrium in RBC enzyme systems. However, this does not exclude the possibility of an antioxidant enzyme imbalance in other tissues.

No differences were observed in RBC α-tocopherol concentrations in DS patients with respect to the control group. These data agree with those of Metcalfe et al. *(33)*, which show that no α-tocopherol deficit exists in the cerebral cortex-mix of DS patients. This may be due to the compensatory effect of the antioxidant enzyme system (GPx, GR, and CAT), which is induced in DS patients, effectively avoiding the consumption of α-tocopherol.

There are discrepancies with regard to the composition of fatty acids in the brain of DS patients *(34–36)*. Our results indicate an increase in the relative proportion of n3 vs n6 PUFAs. Docosahexenoic acid (C22:6n3) in particular was increased, whereas docosatetrenoic acid (C22:4n6) and docosapentenoic acid (C22:5n6) were decreased in the RBC membranes of DS patients vs controls. These results agree with those reported by Brooksbank et al. *(34)* in phospholipids of the fetal cerebral cortex-mix and suggest that the imbalance in PUFA content observed during prenatal development is maintained throughout life in other cell membranes, such as in RBCs. On the other hand, the n6 PUFAs dihomo-γ-linolenic and arachidonic (C20:3n6, C20:4n6) are increased in DS patients. This finding, coupled with a decrease in linoleic acid (C18:2n6), suggests an increase in Δ-6-desaturase activity in these patients. Increases in the activity of other enzyme systems, such as kinases and enzymes of the pentose-phosphate route, have also been described *(37)*. It has been suggested that there may be a general disruption in cell metabolism that might explain the possible increase in desaturase activity. However, these are mere speculations, because Δ-6-desaturase activity was not assessed in the present study. Moreover, other mechanisms, such as increased retroconversion of C22:4n6, could account for the excess of arachidonic acid (C20:4n6) in these patients. In fact, concentrations of these long-chain n6 PUFAs are decreased in our DS patients.

Despite the fact that docosahexenoic acid (C22:6n3) is the most susceptible PUFA to peroxidation because it has the highest number of double bonds, the increase of docosahexenoic acid in DS patients suggests that its content in cell membranes is not influenced substantially by the oxidative action of RO species, probably because of the compensatory increase of the antioxidant enzyme cell systems. Although most authors suggest the existence of an imbalance in the antioxidant cell system *(24, 25, 27, 28)*, others have shown that an increase in SOD1 activity is not necessarily accompanied by an increase in lipid peroxidation *(38)*. Hayn et al. *(39)* did not find evidence for a pathogenic role of RO species in aged patients with DS and Alzheimer's disease.

Our results indicate that the increase in SOD1 activity in the RBCs of DS patients is associated with an adaptive rise in the activity of the H_2O_2-scavenging enzymes GPx and CAT. These results suggest that peroxidative damage would be prevented in RBCs or that, if it occurs, it would be mild and would not affect the fatty acid composition of cell membranes. Therefore, if oxidative stress causes alterations such as atherosclerosis, early cell aging, and neurologic disorders in DS patients, these oxidative alterations are not reflected in erythrocytes, as we can see in our results. Further studies are necessary to fully understand the mechanisms involved in the pathogenesis of these alterations in DS patients.

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