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Original Contribution

INFLUENCE OF AGE ON ACTIVITIES OF ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION PRODUCTS IN ERYTHROCYTES AND NEUTROPHILS OF DOWN SYNDROME PATIENTS

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Abstract—Thirty-seven individuals with Down syndrome (DS) were divided into four age categories: (i) 1 to < 6 years,
(ii) 6 to < 13 years, (iii) 13 to < 20 years, and (iv) over 20 years. Activities of antioxidant enzymes found in individual
age categories were different, but the differences between age groups were not statistically significant. We confirmed
significantly higher activities of Cu/Zn superoxide dismutase (SOD) and glutathione peroxidase (GPx) in blood cells of
people with DS as compared to 35 controls, which consisted, for the first time, of siblings of children with DS. No
significant differences were found in activities of catalase and glutathione reductase in DS vs. controls. A significant
difference was observed in serum concentration of malondialdehyde (MDA) in DS vs. controls (8.39 ± 0.34 μmol/l vs.
7.34 ± 0.27 μmol/l; p = .021) and concentration of MDA in erythrocytes of individuals with DS between the third and
fourth age group (p = .05). In DS persons, an elevated ratio of SOD to catalase plus GPx with respect to the controls
in all age categories was found, suggesting oxidative imbalance, potentially contributing to accelerated aging observed
in these persons.   © 2001 Elsevier Science Inc.

Keywords—Down syndrome, Antioxidant enzymes, Aging, Malondialdehyde, Lipofuscin, Free radicals

INTRODUCTION

Down syndrome (DS) is characterized by trisomy of chromosome 21. The disease is associated with mental retardation, immune system disorders, and autoimmune processes. It is further characterized by increased incidence of heart defects, gastrointestinal anomalies, and malignancies. Typical manifestations of Down syndrome are the onset of premature aging and Alzheimer disease pathology at the age of 30–40 years. Many individuals with Down syndrome suffer from premature dementia [1]. Premature incidence of degenerative free radical pathological conditions, like cataract and autoimmune

disorders, was also described [2–4]. Persons with Down syndrome are more susceptible to respiratory tract and skin infections. Immune system alterations concern, for the most part, cell-mediated immunity and phagocyte functions rather than antibody immunity. Alterations in oxidative metabolism of neutrophils contribute to alterations of physiological functions in these persons.

Individuals with DS are characterized by an excess activity of Cu/Zn-superoxide dismutase (SOD) (EC 1.15.1.1) in all cells by about 50%. SOD together with glutathione peroxidase (GPx) (EC 1.11.1.9), glutathione reductase (GR) (EC 1.6.4.2), myeloperoxidase (MPO) (EC 1.11.1.7), and catalase (CAT) (EC 1.11.1.6) form the main enzyme protection mechanism against harmful effects of reactive oxygen species (ROS). Disturbance of antioxidant enzyme balance in persons with DS can be a key to DS pathogenesis. GPx reduces lipid hydroperoxides, thereby indirectly protecting hydrophobic mem-

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brane compartments. A sufficiently increased GPx activity could be a suitable protection mechanism against peroxidation processes inside cells of DS patients, though expression of this selenoenzyme can be limited by decreased selenium levels, observed in DS. SOD and CAT, on the other hand, primarily operate in hydrophilic environment. A general view of the efficiency of the three antioxidant enzymes (SOD, GPx, CAT), and of the stability of the system according to their physiological and inhibited activity, is discussed by Remacle et al. [5].

Trisomic cells are more sensitive to oxidative stress. This sensitivity may be due to an imbalance in the hydrogen peroxide metabolism or another unknown factor. The cells tend to compensate this negative effect by increasing GPx activity, while that of catalase remains unchanged. Thus there should be changes in the ratio of SOD to (CAT + GPx), which is important from the point of view of sensitivity to ROS, rather than in absolute quantities of individual antioxidants themselves. There are specific synergic interactions between antioxidant enzymes and therefore a comprehensive understanding of the antioxidative system should be based on knowledge of activities and mutual interactions of enzymes, involved in free radical detoxification [6].

Our aim was to monitor accompanying alterations in metabolism of antioxidant enzymes and lipids in persons with DS in age dependence. DS is known to be accompanied by increased activity of Cu/Zn-SOD and, at the same time, immune system disorders. We measured activities of SOD, GPx, CAT, and GR in erythrocytes by standard methods and compared them with activities of the same enzymes and myeloperoxidase (MPO) in neutrophils, in immuno-competent cells with physiologically required superoxide production. We were also interested in the relation of individual antioxidants or their groups to age of persons with DS with regard to accelerated aging processes as one of the important manifestations of DS in adolescents and adults.

MATERIALS AND METHODS

Sample of patients

During the years 1997 to 1999, we examined 37 individuals with Down syndrome, aged from 1.7 to 46.8 years. In all persons with DS, the Down syndrome diagnosis was confirmed by a cytogenetic examination and determined to be nondisjunction trisomy 21 in 36 cases, and Robertsonian translocation in one case. All patients in this study are regularly monitored at the Down Syndrome Department of the Institute for Preventive and Clinical Medicine in Bratislava, Slovakia. For selected examinations, biological samples were taken after a prior written consent of parents.

Individuals with DS suffering from serious heart defects or chronic diseases like diabetes mellitus, children with acute respiration diseases, and couples of siblings where age difference between the child with DS and the other child exceeded 5 years, were excluded from the study.

Control group

The control group was comprised of healthy siblings of monitored persons with DS. In one case we examined dizygotic twins—a girl with DS and her healthy brother without trisomy 21.

One of the selection criteria adopted for this study was family environment and home care for the persons with DS (as opposed to institutional welfare), equal nutritive habits for both siblings (DS and control—diet, consumption of vitamins, etc.) and optimum attitude of the family.

The individuals with DS and their siblings, who formed the control group, were divided into four age groups:

1. 1–<6 years.
2. 6–<13 years.
3. 13–<20 years.
4. Over 20 years.

Blood samples were taken according to the principles of Helsinki declaration.

Body mass index

The Body mass index (BMI) was calculated as follows: BMI = weight/(height)^2 (kg/m^2).

Materials

Glucose-6-phosphate dehydrogenase, chinin sulphate, cumene hydroperoxide, lactoperoxidase, luminol, lysozyme, glutathione, NADP$^+$, NADPH, hydrogen peroxide, Cu/Zn-superoxide dismutase, 1,1,3,3-tetra-ethoxypropane, and tetramethylbenzidine were from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione reductase and peroxidase were from Boehringer Mannheim GmbH (Wien, Austria). Dextrane T-500 was from Pharmacia Fine Chemicals (Uppsala, Sweden). 5,5′-di-thio-bis(2-nitrobenzoic acid) (DTNB) was from Serva (Heidelberg, Germany), glucose-6-phosphate was from Reanal (Budapest, Hungary). Thiobarbituric acid and magnesium chloride hexahydrate were from Merck (Darmstadt, Germany). Heparin was from Spofa (Prague, Czech Republic). The RANSEL kit was from RANDOX (London, UK) and SOD kit from F.A.T. (Berlin, Germa-

ny). Other chemicals were from Lachema (Brno, Czech Republic). All chemicals used were of p. a. grade.

Isolation of neutrophils

Neutrophils were isolated from heparinized venous blood (25 U/ml) using a modification of the Boyum method in 6% dextrane [7]. Remaining erythrocytes were damaged by hypotonic lysis with cold distilled water for 10 s. Isotonicity was restored using a 0.6 mol/l NaCl solution. The neutrophils suspension was finally diluted in phosphate buffer (PBS), pH 7.4 to concentration of 3×10^7 neutrophils/ml. Differential counting was done using the Giems-Romanovsky solution. The resulting cell suspension contained at least 90% neutrophils. After subsequent freezing to $-20°C$, the suspension was sonicated (UG 160/320 TA, TESLA, Czech Republic) in ice-water bath four times for 20 s and centrifuged at $22,000 \times g$ for 30 min. The sonication is controlled every time under the scope. The cytoplasmic fraction was used to determine activity of antioxidant enzymes. Activities of GPx, GR, CAT, and MPO were expressed in specific activity units—katals per milligram of total protein present in cytosol (for neutrophils), or per gram of hemoglobin (for erythrocytes). Katal (kat) is defined as catalytic activity, whereby one mole of substrate is metabolized per enzyme molecule per one second (mol/s). Protein content in the cytoplasmic fraction was determined by the Lowry method, using lysozyme as a standard.

Isolation of erythrocytes for determination of superoxide dismutase

Erythrocytes (0.5 ml) were washed three times with a 0.15 mol/l NaCl solution. After centrifugation ($400 \times g$, 5 min) they were resuspended in a 0.15 mol/l NaCl solution and sonicated for 5 s at $4°C$. Hemoglobin concentration was measured in hemolysate using the Drabkin agent. Subsequently, hemoglobin was removed from the hemolysate by extraction in a chloroform-ethanol mixture (3:5) (I. Popov, personal communication). The upper layer was used for determination of superoxide dismutase activity.

Isolation of erythrocytes for determination of other enzymes

Heparinized venous blood (25 U/ml) was washed three times with a 0.15 mol/l NaCl solution. After centrifugation ($400 \times g$, 5 min), the erythrocytes were hemolyzed by adding triple volume of distilled water.

Hemoglobin concentration in the hemolysate was measured using the Drabkin method.

Determination of enzymes

Superoxide dismutase (SOD) activity was determined by a photochemiluminescence method using luminol. We used the commercial kit for SOD (F.A.T., Berlin) using bovine Cu/Zn-SOD (Sigma) as a standard. The method is based on photochemical production of free radicals combined with their photochemiluminescence detection [8] (PHOTOCHEM photochemiluminometer, F.A.T.). SOD activity was expressed in mg SOD/g Hb for erythrocytes and in μg SOD/mg proteins in the cytoplasmic fraction of neutrophils.

Glutathione peroxidase in neutrophils was determined using the modified method of Blanchflower [9]. The reaction mixture (0.6 ml) contained, in final concentration, 1.54 mmol/l GSH, 0.192 mmol/l NADPH, 30.76 U/l GR in phosphate buffer (0.04 mol/l), pH 7.0, with 8 mmol/l EDTA, and 50 μl of a sample. The reaction was initiated by addition of cumene hydroperoxide to the final concentration of 3.4 mmol/l. The reaction rate was determined from a decrease of absorbance at 340 nm at $37°C$ (Labsystem Oy, FP-901, Chemistry Analyse, Helsinki, Finland). GPx activity was expressed in kat/mg of protein.

Glutathione peroxidase in erythrocytes was determined using the commercial RANSEL kit (RANDOX), a coupled assay with glutathione reductase, using cumene hydroperoxide as substrate, measuring the decrease in absorbance at 340 nm. GPx activity was expressed in kat/g Hb.

Glutathione reductase was measured using the method of Delides [10]. Fifty μl of $10 \times$ diluted cytoplasmic fraction (or hemolysate) was incubated for 15 min at $30°C$ in a solution, containing 0.295 mmol/l glucose-6-phosphate, 0.164 mmol/l $NADP^+$, 4.39 mmol/l $MgCl_2$, and 125 U/l glucose-6-phosphate dehydrogenase in phosphate buffer (0.125 mol/l), pH 7.5 with 6.3 mmol/l EDTA. The reaction was initiated by adding 1.49 mmol/l GSSG and stopped by incubation in a boiling water bath (2 min). After centrifugation ($3500 \times g$, 10 min), the supernatant was used for determination of reduced glutathione with Ellman's reagent (DTNB) (Labsystem Oy, FP-901, Chemistry Analyse). GR activity was expressed in kat GSH/mg of cytoplasmic protein or in kat GSH/g of Hb.

Catalase was determined using the method of Cavarocchi [11]. One ml of reaction mixture contained 1 mmol/l H_2O_2 in phosphate buffer (0.01 mol/l), pH 7.8, and a sample or water. The mixture was incubated for 10 min at room temperature and the reaction was stopped by addition of a solution, containing 1.476 mmol/l 4-aminoantipyridin

Table 1. Clinical and Biochemical Characteristics of Patients

| Parameter | Mean ± SEM (n) | | Sign. *p* |
	Down syndrome	Control	
Clinical and biochemical characteristics of patients			
Age (years)	14.8 ± 1.6 (37)	13.0 ± 1.3 (35)	.485
BMI (kg/m^2)	21.7 ± 1.0 (37)	18.8 ± 0.7 (35)	.067
Cholesterol (mmol/l)	4.61 ± 0.15 (33)	4.41 ± 0.13 (30)	.487
Triglyceride (mmol/l)	1.30 ± 0.13 (26)	0.83 ± 0.09 (24)	.007
Activities of antioxidant enzymes in neutrophils			
SOD (μg/mg P)	8.43 ± 0.46 (31)	6.12 ± 0.38 (28)	.001
GPx (nkat/mg P)	1.52 ± 0.11 (32)	1.11 ± 0.09 (27)	.012
GR (nkat/mg P)	1.60 ± 0.10 (29)	1.69 ± 0.11 (30)	.391
CAT (nkat/mg P)	103.2 ± 7.4 (29)	99.9 ± 5.8 (29)	.762
MPO (U/mg P)	3.44 ± 0.19 (23)	3.38 ± 0.29 (30)	.409
Activities of antioxidant enzymes in erythrocytes			
SOD (mg/g Hb)	0.82 ± 0.05 (29)	0.52 ± 0.03 (25)	<.001
GPx (μkat/g Hb)	0.95 ± 0.06 (37)	0.72 ± 0.04 (30)	.004
GR (nkat/g Hb)	66.1 ± 4.2 (36)	58.5 ± 3.2 (32)	.390
CAT (μkat/g Hb)	123.9 ± 7.7 (32)	128.7 ± 7.5 (29)	.461
Levels of oxidative degradation products			
MDA-E (μmol/g Hb)	2.20 ± 0.09 (37)	2.09 ± 0.08 (33)	.355
MDA-S (μmol/l)	8.39 ± 0.34 (31)	7.34 ± 0.27 (30)	.021
Lipofuscin (μmol/l)	7.98 ± 0.60 (27)	7.70 ± 0.37 (27)	.672
Ratios of antioxidant enzymes			
R_1 (neutrophils)	0.095 ± 0.008 (16)	0.059 ± 0.005 (19)	<.001
R_2 (erythrocytes)	0.0076 ± 0.0006 (24)	0.0044 ± 0.0003 (19)	<.001

Significance levels *p* are based on between-group (DS vs. controls) comparison using the Mann-Whitney U-test. Data are given as Mean ± SEM (*n*), *n* is the number of subjects per group; MDA-E = erythrocyte MDA; MDA-S = serum MDA; R_1 = SOD/(GPx + CAT + MPO) (activities of enzymes in neutrophils); R_2 = SOD/(GPx + CAT) (activities of enzymes in erythrocytes).

in phosphate buffer (0.1 mol/l), pH 7.0, 1250 U/l peroxidase and 0.05% phenol. Absorbance of the reaction product was measured at 505 nm (spectrophotometer PM 2 DL, Opton, Jena, Germany). The activity was expressed in kat/mg of cytoplasmic proteins or in kat/g of Hb.

Myeloperoxidase was determined using a modified method according to Suzuki et al. [12]. The reaction mixture (100 μl) contained: 10 μl of 6 × diluted cytoplasmic fraction, 0.164 mmol/l tetramethyl benzidine in phosphate buffer, pH 6.0. The reaction was initiated by adding hydrogen peroxide (0.5 mmol/l), and after 20 min incubation, the reaction was stopped by addition of sulphuric acid. Absorbance of the reaction product was measured at 460 nm (spektrophotometer PM 2 DL, Opton). Activity was expressed in U/mg cytoplasmic protein. The unit U is defined as the quantity of enzyme that produces 1.0 ng purpurogallin from pyrogallol within 20 s at pH 6.0 and 20°C.

Lipid peroxidation. A product of lipid peroxidation, malondialdehyde, was determined using the reaction with thiobarbituric acid (TBA) [13]. Amplification of peroxidation during the assay was prevented by the addition of the chain-breaking antioxidant BHT (10 μl of 2 mmol/l per 1 ml of blood) to the heparinized blood. Concentration of thiobarbituric acid reactive products was expressed as malondialdehyde (mol/l) using 1,1,3,3-tetraethoxypropane as standard.

Lipofuscin. Fluorescence determination of lipofuscin is based on the yellow autofluorescence of lipofuscin when excited by UV light (345 nm), that we measured at 430 nm (spectrofluorimeter AMINCO-BOWMAN, Silver Spring, MD, USA) [14]. Lipofuscin concentration in serum was expressed in mol/l, where chinin sulphate was used as a standard.

Statistical analysis

All results are presented as mean ± standard error (SEM) along with sample sizes. Between-group comparisons were made using the Mann-Whitney U-test and analysis of variance (ANOVA) with Tukey post-hoc comparisons. We consider "statistically significant" those differences, for which the respective test gave *p* < .05. Correlations were expressed using the Pearson's (product-moment) correlation coefficient.

RESULTS

Basic clinical parameters of individuals with Down syndrome (DS) and healthy individuals (C) are listed in Table 1. No statistically significant differences were found in age composition of the DS group and control group in either age category. In the values of body mass index (BMI), there is a statistically marginally significant
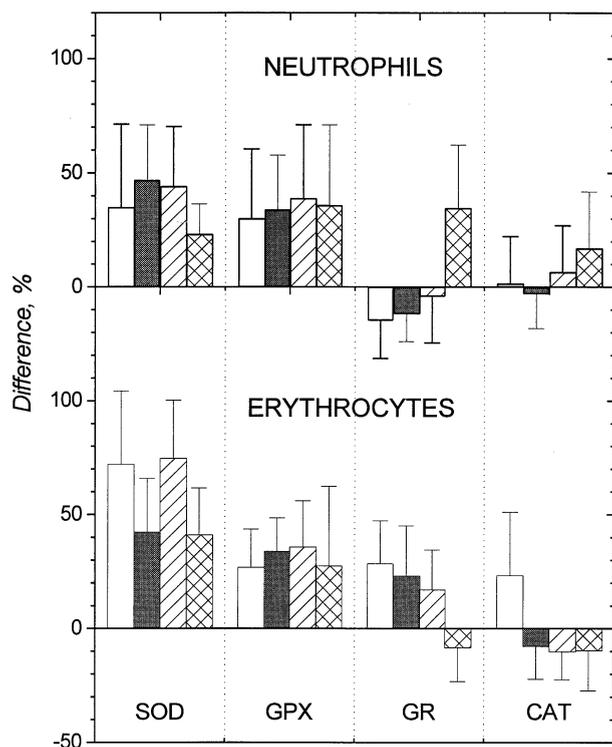
Fig. 1. Activities of antioxidant enzymes in neutrophils and erythrocytes of persons with Down syndrome by age categories. Enzyme activities are reported as percent deviations from the values for the control group. Individual bars correspond to age categories: white = 1 to 6 years; black = 6 to 13 years; //// = 13 to 20 years; XXX = over 20 years. Error bars indicate standard errors.
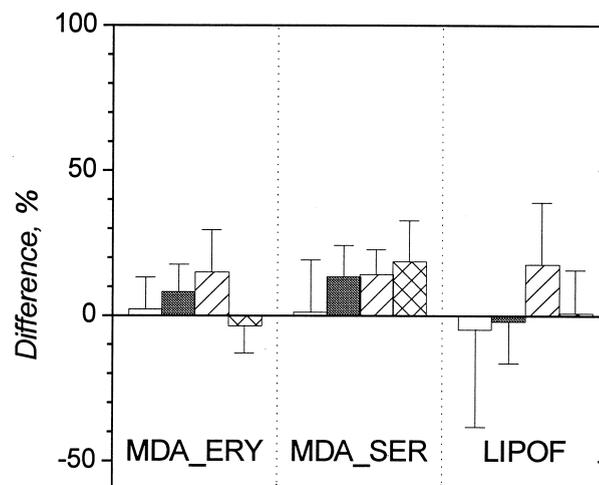

Fig. 2. Levels of oxidative damage products in persons with Down syndrome by age categories. Concentrations are reported as percent deviations from the values for the control group. Individual bars correspond to age categories: white = 1 to 6 years; black = 6 to 13 years; //// = 13 to 20 years; XXX = over 20 years. Error bars indicate standard errors.

difference between the DS and C groups ($p = .067$). There is a significant difference between DS and C in the 13–20 age group ($p = .002$) and a marginally significant difference in the over-20 age group ($p = .068$). There was no statistically significant difference in cholesterol levels, while there was a significant difference in the values of triglycerides between the DS and C group for the whole groups. No significant gender differences were found.

Activities of antioxidant enzymes (SOD, GPx, GR, CAT) in erythrocytes and neutrophils and MPO in neutrophils for individual age groups are listed in Table 1 and are shown for individual age categories in Fig. 1. The results in Table 1 confirm statistically significant differences in activities of SOD and GPx between persons from the DS and C groups both in erythrocytes and neutrophils, if division into individual age categories is disregarded. At the level of individual age categories, only marginally significant differences between the DS and control group were observed in SOD activity in neutrophils ($p = .05$ to $.10$). GPx activity was not significantly different in DS compared to the controls at the level of individual age groups. For SOD activity in erythrocytes, at the level of age groups, significant dif-

ferences were found only for age groups 1–6 and 13–20, and a marginally significant difference ($p = .063$) was found for the over-20 age group.

For GPx in erythrocytes, a significant difference was found only for the 6–13 age group, and a marginally significant difference ($p = .077$) for the 13–20 age group (Fig. 1). No significant differences between individual age groups for SOD and CAT activities of DS and controls were found. A significant decrease between the over-20 and 6–13 age groups ($p = .027$) was found in GR activity of neutrophils (Fig. 1).

No statistically significant differences between the DS group and control group were found in activities of other enzymes studied, either at the level of the whole groups or at the level of individual age categories (Fig. 1).

Concentrations of lipid peroxidation products, serum and erythrocyte malondialdehyde and serum lipofuscin are shown in Table 1 and in Fig. 2. At the whole group level, the difference in serum MDA concentration between individuals from the DS and control groups is statistically significant. No significant differences were found in individual age categories, though the differences in relative MDA concentration to the controls increase with age (Fig. 2). No statistically significant differences between the DS and control groups were found either in MDA concentration in erythrocytes or in serum lipofuscin concentration.

In individuals with DS, accelerated aging is supposed to arise as a result of potential oxidative stress. For this reason, we investigated correlations between activities of antioxidant enzymes and age.

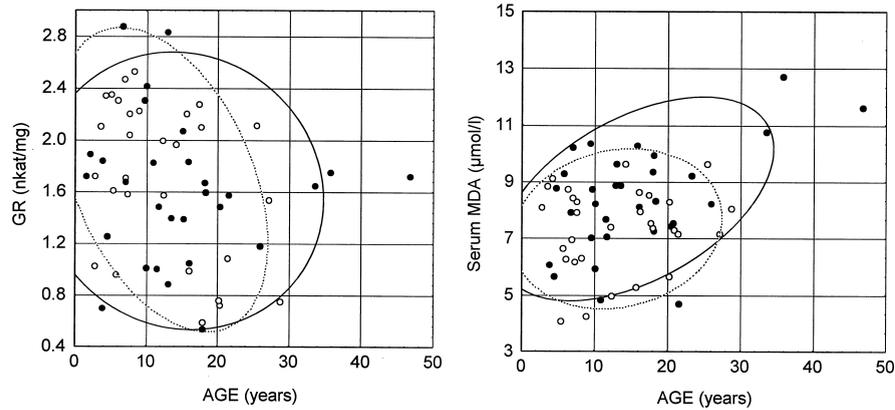SOD activities in neutrophils and in erythrocytes, as

Fig. 3. Left: Correlation between age and activity of glutathione reductase in neutrophils of persons with Down syndrome ($\bullet$, solid ellipse; $r = -0.065$, $p = .73$, $n = 29$) and controls ($\bigcirc$, dotted ellipse; $r = -0.419$, $p = .021$, $n = 30$). Right: Correlation between age and MDA level in serum of persons with DS ($\bullet$, solid ellipse; $r = 0.49$, $p = .005$, $n = 31$) and controls ($\bigcirc$, dotted ellipse; $r = 0.14$, $p = .47$, $n = 30$). Ellipses indicate 95% confidence regions for data.

well as activities of the other enzymes under study in the DS and control group, do not change significantly with age. There is only one exception—GR in neutrophils, which decreases significantly with age in controls ($r = -0.419$, $p = .021$), whereas the activity in the DS group was age independent (Fig. 3).

The concentration of oxidation product MDA in serum was found to increase significantly with age in the DS group ($r = 0.49$, $p = .005$), but not in the control group (Fig. 3). No significant relation between levels of oxidative damage products (MDA, lipofuscin) and individual antioxidant enzymes in neutrophils or erythrocytes was found. The only exception is CAT; we found that erythrocyte MDA levels show significant negative correlation with CAT activity in neutrophils ($r = -0.377$, $p = .044$ for DS and $r = -0.304$, $p = .02$ for DS and controls combined) (Fig. 4).

Changes in the balance between hydrogen peroxide production from superoxide in the dismutation reaction catalyzed by SOD and its decomposition by other enzymes (GPx, CAT, and MPO in neutrophils, and GPx and CAT in erythrocytes) can be expressed using the ratio of activities of SOD/(GPx + CAT + MPO) for neutrophils (R_1), and SOD/(GPx + CAT) for erythrocytes (R_2). Both values are significantly different in persons with DS and in the control group ($p < .001$, Table 1). The differences in the R_1 and R_2 ratios between controls and DS subjects in age dependence are shown in Fig. 5.

In the over-20 age group, the ratio R_1 decreases relative to controls. This correlates with decreasing activity of SOD in this age group (Fig.1). In erythrocytes, we have not observed any change of the R_2 ratio with age.

In this study, we also monitored correlations between the R_1 ratio and MDA. We have found a significant correlation between R_1 and erythrocyte MDA for joined

group ($r = 0.44$, $p = .01$) and controls ($r = 0.48$, $p = .04$), although for the DS group the correlation was insignificant ($r = 0.2$, $p = .46$) (Fig. 6, left). Between R_2 and serum MDA we have found positive correlation ($r = 0.41$, $p = .01$) for the joined group and marginally significant correlation ($r = 0.40$, $p = .07$) for the DS group (Fig. 6, right). Neither the R_1 nor R_2 ratio has a significant effect on lipofuscin level.

DISCUSSION

It has been generally accepted that individuals with Down syndrome are exposed to an excessive oxidative
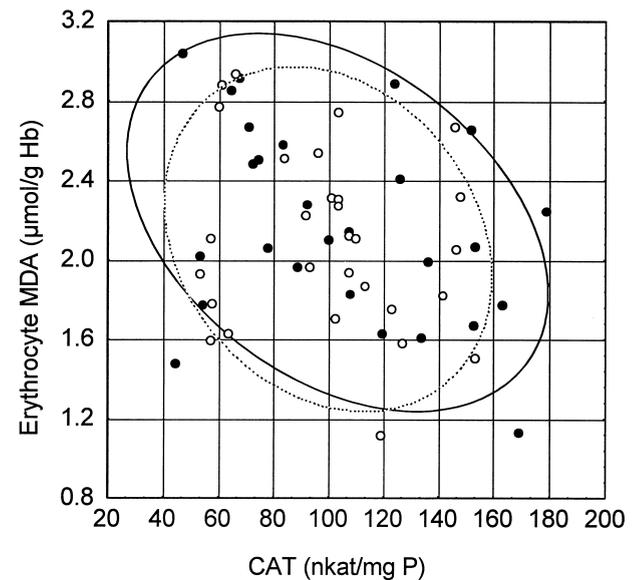

Fig. 4. Correlation between CAT activity and MDA level in erythrocytes of persons with DS ($\bullet$, solid ellipse; $r = -0.38$, $p = .04$, $n = 28$) and controls ($\bigcirc$, dotted ellipse; $r = -0.22$, $p = .26$, $n = 29$). Ellipses indicate 95% confidence regions for data.
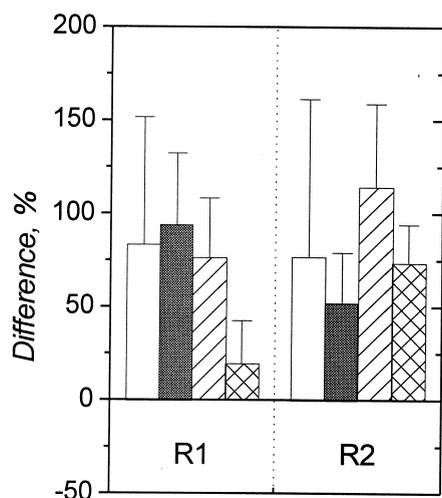
Fig. 5. Ratio of antioxidant enzymes. R_1 = SOD/(GPx + CAT + MPO) (activities of enzymes in neutrophils) and R_2 = SOD/(GPx + CAT) (activities of enzymes in erythrocytes) for persons with Down syndrome by age categories. Values are expressed as percent deviations from the values of the control group. Individual bars correspond to age categories: white = 1 to 6 years; black = 6 to 13 years; //// = 13 to 20 years; XXX = over 20 years. Error bars indicate standard errors.

H_2O_2, substrate for GPx. Increased activities of SOD and GPx found in erythrocytes and neutrophils of persons with DS are consistent with the results of other studies [18,19,21,22]. The increased GPx activity found in this study requires a sufficient amount of GSH. However, a significant lowering of GSH level in persons with DS as compared with the controls was found [23].

In accordance with other studies [24,25], we have not observed changes in the activity of catalase, another enzyme that decomposes hydrogen peroxide.

Sensitivity of a cell to free radicals apparently depends on the relationship between SOD and (CAT + GPx) rather than on absolute amounts of individual antioxidants [6]. In cells of persons with DS, the ratio of SOD to enzymes that inactivate hydrogen peroxide is higher than in the cells of healthy persons from the control group (Fig. 5, Table1). This imbalance could support accumulation of hydrogen peroxide in cells. Amstad et al. [26] have found that epidermal cells with overproduction of SOD are very sensitive to superoxide and hydrogen peroxide, while cells with overproduction of CAT are protected against effects of oxidants.

The potential oxidative stress caused by excess of SOD can be caused by:

- Lack of superoxide, which inhibits termination reaction of lipid hydroperoxide radical cycle, and thus leads to increase in their concentration.
- High concentration of H_2O_2, which stimulates autooxidation of hydroquinons, while concurrent lack of superoxide inhibits termination reaction of the semiquinon radical [6].
- Increased concentration of hydrogen peroxide, which inhibits some protection enzymes, thereby allowing production of $^{\bullet}OH$, especially in the presence of catalytically active metals [27,28]. The hydroxyl
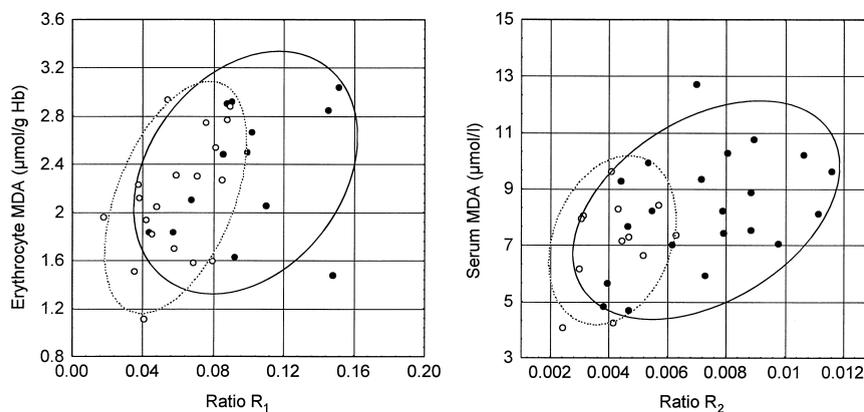
stress arising from the presence of three chromosomes 21 [15]. The excessive genetic dose of Cu/Zn superoxide dismutase gives rise to an increased SOD activity, which can have key importance in the pathogenesis of Down syndrome [16,17]. Increased activity of SOD apparently leads to an imbalance of reactive oxygen species in the body, which brings about extensive secondary effects on metabolism [18].

The increased activity of GPx apparently results from a secondary regulatory mechanism [19]. The gene for GPx is coded by chromosome 3 [20]. Increased activity of GPx is most likely induced by increased levels of

Fig. 6. Correlation between the ratio of activities of enzymes and MDA. Left: R_1 = SOD/(GPx + CAT + MPO) in neutrophils and MDA level in erythrocytes in persons with DS ($\bullet$, solid ellipse; r = 0.20, p = .46, n = 16) and controls ($\bigcirc$, dotted ellipse; r = 0.403, p = .04, n = 19). Right: R_2 = SOD/(GPx + CAT) in erythrocytes and MDA in serum of persons with DS ($\bullet$, solid ellipse; r = 0.40, p = .07, n = 21) and controls ($\bigcirc$, dotted ellipse; r = 0.28, p = .27, n = 17). Ellipses indicate 95% confidence regions for data.

radical damages various cellular structures (proteins, lipids, DNA, RNA), alters nitrogen-containing hetero-cyclic bases, and induces cross-linking and strand breaks.

Paradoxically, hydrogen peroxide, as a product of dismutation reaction on the one hand, can, on the other hand, be a substrate for the same enzyme. Jewett et al. [29] have recently discussed the possible peroxidase activity of Cu/Zn-SOD. They summarized that the release of Cu(I) and 2-oxo-histidine formation is followed by the reaction of SOD with hydrogen peroxide to form a non-enzyme-bound oxidant.

The potential adverse effect associated with overdoses of SOD in cell-free experimental system was studied by Offer et al. [30]. They concluded that the most significant outcome of higher SOD concentration is a respective decrease in $[O_2^{\bullet -}]_{steady\ state}$ rather than any significant elevation of $[H_2O_2]_{steady\ state}$. At high SOD concentrations, and therefore ultra-low $[O_2^{\bullet -}]_{steady\ state}$, the SOD in its higher oxidation state (Cu(II)/Zn-SOD), not only reacts with $O_2^{\bullet -}$., but also attacks and oxidizes the target molecule for which it was supported to provide protection.

Physiological consequences resulting from the over-expression of Cu/Zn-SOD have been studied by Groner et al. [31] on cellular and animal models. The tongue neuromuscular junctions (NMJ) in transgenic mice exhibited significant withdrawal and destruction of some terminal axons and the development of multiple small terminals. The morphological changes in the transgenic NMJ mice were similar to those previously seen in muscles of aging mice and rats, as well as in tongue muscle of persons with Down syndrome. The findings by these authors suggest that Cu/Zn-SOD gene dose is involved in the pathological abnormalities of tongue NMJ observed in Down syndrome patients.

Oxidative stress conditions [32] are further confirmed by increased levels of a lipid oxidation damage product, serum malondialdehyde, found in this study, in line with the literature [18,24,33]. Likewise, there are elevated levels of 8-hydroxy-2-deoxyguanosine [34]. However, DNA strand breaks could not be detected by single cell electrophoresis (comet assay) in persons with DS (Šustrová, unpublished results).

Accelerated aging in persons with DS is supposed to originate as a result of oxidative stress. With the exception of GR in control subjects, no significant changes in activities of antioxidant enzymes under study were found. This finding correlates with the results of Omata et al. [35]. Our results demonstrate that accelerated aging of persons with Down syndrome is probably not primarily related to activities of individual antioxidant enzymes.

Niwa et al. [36], who, in accordance with our results, observed unchanged SOD activity in neutrophils of healthy individuals in age dependence, determined a decrease in inducibility of the enzyme after the age of 40. They attributed the more frequent incidence of infectious diseases in older nontrisomic persons to this finding. Niwa et al. [37] determined activities of other enzymes of oxygen metabolism—GPx and CAT in neutrophils, finding a decrease with age, but no difference in activity after induction.

Despite the elevation of GPx activity in neutrophils of DS persons we have found lower GR activity, especially in lower age groups (below 20 years). A significant decrease in GR activity between age groups 4 and 2 was seen for DS and controls combined ($p = .027$) (Fig. 1). This can lead to imbalance of GSH metabolism of DS individuals published also in our previous work [23].

Absence of correlation of SOD, GPx, and CAT in both cells and MPO in neutrophils with age in our DS group can be explained by the relatively low number of older DS patients (range: 1.7 to 46.8 years, mean $\pm$ SEM: 14.8 ± 9.7 years). We obtained insignificant differences in enzymatic activities between individual age groups, and correlation of enzymatic activities with age was found to be insignificant, too.

The products of lipid peroxidation (MDA in erythrocytes and lipofuscin) were not significantly changed in the DS group compared to the controls. However, MDA in serum of persons with DS was significantly elevated (Table 1) and a significant correlation between MDA in serum and age in the DS group (Fig. 3, $r = 0.49$, $p = .005$) was found.

No significant correlation was found for MDA in serum and individual antioxidant enzymes. Similarly, the correlation between MDA in erythrocytes and SOD, GPx, GR, and MPO was not significant. Only catalase exhibited a negative correlation to MDA in erythrocytes ($r = -0.377$, $p = .044$ for DS and $r = -0.304$, $p = .02$ for all group) (Fig. 4).

At present, the cause of increased oxidative stress in persons with DS is unclear. Remacle et al. [5] found that the ratio of activity of SOD to that of GPx, which gives the same protection, varies from 246 to 1008, according to the severity of the stress. Changes in equilibrium between formation of hydrogen peroxide from superoxide in dismutation reaction catalyzed by SOD and its decomposition by other enzymes (GPx, CAT, and MPO in neutrophils, and GPx and CAT in erythrocytes) are expressed by the ratios of activities of SOD/(GPx + CAT + MPO) for neutrophils (R_1) and SOD/(GPx + CAT) for erythrocytes (R_2). The observed increase of serum MDA concentration in the DS group (Table 1) could be one of the consequences of imbalance of the activities of antioxidant enzymes. In the joined group,

the increase in both R_1 ($p = .01$) and R_2 ($p = .01$) ratios significantly correlates with increased production of MDA. MDA in erythrocytes negatively correlates with catalase in the joined groups ($r = -0.304$, $p = .02$) and in the DS group ($r = -0.377$, $p = .044$). This finding is in concordance with the ascertained imbalance in relative ratio of antioxidant enzymes in Down syndrome patients.

In summary, our data are consistent with previous evidence of the imbalance in activities of individual antioxidant enzymes in Down syndrome patients. On the basis of correlation analyses between antioxidant enzymes and some products of lipoperoxidation, we concluded that the oxidative imbalance in trisomic cells is age dependent and depends on the relationship between SOD and (CAT + GPx) rather than on absolute amounts of individual antioxidant enzymes.

REFERENCES

[1] Kolata, G. Down syndrome—Alzheimer's linked. *Science* **230:**1152–1156; 1985.

[2] de Haan, J. B.; Wolvetang, E. J.; Cristiano, F.; Iannello, R.; Bladier, C.; Kelner, M.; Kola, I. Reactive oxygen species and their contribution to pathology in Down syndrome. *Adv. Pharmacol.* **38:**379–385; 1997.

[3] Simonian, N. A.; Coyle, J. T. Oxidative stress in neurodegenerative disease. *Annu. Rev. Pharmacol.Toxicol.* **36:**83–91; 1996.

[4] Cohen, W. I. Health care guidelines for individuals with Down syndrome: 1999 revision. *Down Syndr. Q.* **4:**1–15; 1999.

[5] Remacle, J.; Lambert, D.; Raes, M.; Pigeolet, E.; Michiels, C.; Toussaint, D. Importance of various antioxidant enzymes for cell stability. *Biochem. J.* **286:**41–46; 1992.

[6] Michiels, C.; Raes, M.; Toussaint, O.; Remacle, J. Importance of Se-Glutathione Peroxidase, Catalase and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic. Biol. Med.* **17:**235–248; 1994.

[7] Boyum, A. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest. Suppl.* **21:**77–89; 1968.

[8] Popov, I. N.; Lewin, G.; von Baehr, R. Photochemiluminescent detection of antiradical activity. I. Assay of superoxide dismutase. *Biomed. Biochim. Acta* **46:**775–779; 1987.

[9] Blanchflower, W. J.; Rice, D. A.; Davidson, W. B. Blood glutathione peroxidase. A method for measurement and the influence of storage, cyanide, and Drabkin's reagent on enzyme activity. *Biol. Trace Elem. Res.* **11:**89–100; 1986.

[10] Delides, A.; Spooner, R. J.; Goldberg, D. M.; Neal, F. E. An optimized semi-automatic rate method for serum glutathione reductase activity and its application to patients with malignant disease. *J. Clin. Pathol.* **29:**73–77; 1976.

[11] Cavarocchi, N. C.; England, M. D.; O'Brien, J. F.; Solis, E.; Russo, P.; Schaff, H. V.; Orszulak, T. A.; Pluth, J. R.; Kaye, M. P. Superoxide generation during cardiopulmonary bypass: is there a role for vitamin E? *J. Surg. Res.* **40:**519–527; 1986.

[12] Suzuki, K.; Ota, H.; Sasagawa, T.; Sakatani, T.; Fujikura, T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.* **132:**345–351; 1983.

[13] Uchiyama, M.; Mihara, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **86:**271–278; 1978.

[14] Tsuchida, M.; Miura, T.; Mizutani, K.; Aibara, K. Fluorescent substances in mouse and human sera as a parameter of *in vivo* lipid peroxidation. *Biochim. Biophys. Acta* **834:**196–204; 1987.

[15] Ďuračková, Z. *Free radicals and antioxidants in medicine (I)* (in Slovak). Bratislava, Slovakia: Slovak Academic Press; 1998.

[16] Wong, G. H. W.; Pingham, J. L. Tumor necrosis factor protect against oxidative insult: role of manganese superoxide dismutase. In: Aruoma, O. I.; Halliwell, B., eds. *Molecular biology of free radicals in human diseases.* London: OICA International; 1998:397–410.

[17] Garaiová, I. The role of free radicals in heredity diseases (Down syndrome). In: Ďuračková, Z.; Bergendi, L.; Čársky, J., eds. *Free radicals and antioxidants in medicine (II)* (in Slovak). Bratislava, Slovakia: Slovak Academic Press; 1999:263–286.

[18] Kedziora, J.; Bartosz, G. Down's syndrome: a pathology involving the lack of balance of reactive oxygen species. *Free Radic. Biol. Med.* **4:**317–330; 1988.

[19] Néve, J.; Sinet, P. M.; Molle, L.; Nicole, A. Selenium, zinc and copper in Downs syndrome (trisomy 21): blood levels and relations with glutathione peroxidase and superoxide dismutase. *Clin. Chim. Acta* **133:**209–214; 1983.

[20] Björkstén, B.; Back, O.; Gustavson, K. H.; Hallmans, G.; Hagglof, B.; Tarnvik, A. Zinc and immune function in Down's syndrome. *Acta Paediatr. Scand.* **69:**183–187; 1980.

[21] Dickinson, M. J.; Singh, I. Down's syndrome, dementia, and superoxide dismutase. *Br. J. Psychiatry* **162:**811–817; 1993.

[22] Bartosz, G.; Kedziora, J.; Retelewska, W. Decreased oxidant-induced proteolysis in erythrocytes with enhanced antioxidative defence enzymes due to Down's syndrome. *Clin. Chim. Acta* **198:**239–244; 1991.

[23] Ďuračková, Z.; Garaiová, I.; Muchová, J.; Liptáková, A.; Šustrová, M. Metabolism of glutathione at the persons with Down syndrome. *Free Radic. Biol. Med.* **27**(Suppl.):S120; 1999.

[24] Crosti, N.; Bajer, J.; Gentile, M.; Resta, G.; Serra, A. Catalase and glutathione peroxidase activity in cells with trisomy 21. *Clin. Genet.* **36:**107–116; 1989.

[25] Kedziora, J.; Blaszczyk, J.; Sibinska, E.; Bartosz, G. Down's syndrome: increased enzymatic antioxidative defence is accompanied by decreased superoxide anion generation. *Hereditas* **113:**73–75; 1990.

[26] Amstad, P.; Peskin, A.; Shah, G.; Mirault, M. E.; Moret, R.; Zbinden, I.; Cerutti, P. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* **30:**9305–9313; 1991.

[27] Yim, M. B.; Chock, P. B.; Stadtman, E. R. Enzyme function of copper, zinc superoxide dismutase as a free radical generator. *J. Biol. Chem.* **268:**4099–4107; 1993.

[28] Peled-Kamar, M.; Lotem, J.; Wirguin, I.; Weiner, L.; Hermalin, A.; Groner, Y. Oxidative stress mediates impairment of muscle function in transgenic mice with elevated level of wild-type Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **94:**3883–3890; 1997.

[29] Jewett, S. L.; Rocklin, A. M.; Ghanevati, M.; Abel, J. M.; Marach, J. A. A new look at a time-worn system: oxidation of CuZn-SOD by H_2O_2. *Free Radic. Biol. Med.* **26:**905–918; 1999.

[30] Offer, T.; Russo, A.; Samuni, A. The pro-oxidative activity of SOD and nitroxide SOD mimics. *FASEB J.* **14:**1215–1223; 2000.

[31] Groner, Y.; Elroy-Stein, O.; Avraham, K. B.; Schickler, M.; Knobler, H.; Minc-Golomb, D.; Bar-Peled, O.; Yarom, R.; Rotschenker, S. Cell damage by excess CuZnSOD and Down's syndrome. *Biomed. Pharmacother.* **48:**231–240; 1994.

[32] Sies, H., ed. *Oxidative stress: oxidants and antioxidants*. London: Academic Press; 1991.

[33] Percy, M. E.; Dalton, A. J.; Markovic, V. D.; Crapper McLachlan, D. R.; Hummel, J. T.; Rusk, A. C. M.; Andrews, D. F. Red cell superoxide dismutase, glutathione peroxidase and catalase in Down syndrome patients with and without manifestations of Alzheimer disease. *Am. J. Med. Genet.* **35:** 459–467; 1990.

[34] Jovanovic, S. V.; Clements, D.; MacLeod, K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radic. Biol. Med.* **25:**1044–1048; 1998.

[35] Omata, F.; Nakazawa, H.; Nakano, M.; Arimori, S. Erythrocyte superoxide dismutase in various hematological diseases. *Tokai J. Exp. Clin. Med.* **15:**99–106; 1990.

[36] Niwa, Y.; Ishimoto, K.; Kanoh, T. Induction of superoxide dismutase in leukocytes by paraquat: correlation with age and possible predictor of longevity. *Blood* **76:**835–841; 1990.

[37] Niwa, Y.; Iizawa, O.; Ishimoto, K.; Akamatsu, H.; Kanoh, T. Age-dependent basal level and induction capacity of copper-zinc and manganese superoxide dismutase and other scavenging enzyme activities in leukocytes from young and elderly adults. *Am. J. Pathol.* **143:**312–320; 1993.

ABBREVIATIONS

BHT—butylated hydroxytoluene
BMI—body mass index
CAT—catalase
DS—Down syndrome
DTNB—5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent)
EDTA—ethylene diaminetetraacetic acid
GPx—glutathione peroxidase
GR—glutathione reductase
GSH—glutathione
GSSG—glutathione disulfide
Hb—hemoglobin
kat—katal
MDA—malondialdehyde
MPO—myeloperoxidase
NADPH—nicotinamide adeninedinucleotide phosphate (reduced)
NADP$^+$—nicotinamide adeninedinucleotide phosphate (oxidized)
PBS—phosphate-buffered saline
SOD—Cu/Zn-superoxide dismutase
TBA—thiobarbituric acid