The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome

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GARAIOVÁ, I., MUCHOVÁ, J., ŠUSTROVÁ, M., BLAŽÍČEK, P., SIVOŇOVÁ, M., KVASNIČKA, P., PUESCHEL, S. & ĎURAČKOVÁ, Z., The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome. Biologia, Bratislava, **59:** 787—794, 2004; ISSN 0006-3088. (Biologia). ISSN 1335-6399 (Biologia. Section Cellular and Molecular Biology).

Individuals with Down syndrome (DS) suffer from elevated oxidative stress as a consequence of the presence of three copies of chromosome 21, which code for the antioxidant enzyme Cu/Zn superoxide dismutase (SOD). The aim of our study was to gain more complete information about the relationships between antioxidant enzymes, markers of lipoperoxidation and low molecular weight antioxidants. The activities of SOD, glutathione peroxidase (GPx), catalase (CAT) and the concentration of glutathione (GSH), vitamin E, uric acid (UA), total antioxidant status (TAS), malondialdehyde (MDA) and lipofuscin were determined in 44 individuals with Down syndrome and compared with 26 controls. The increased activity of SOD and GPx in erythrocytes of persons with DS was confirmed. No significant difference in the activity of erythrocyte CAT was found between DS and control groups. The ratio of activities of antioxidant enzymes $R = SOD/(GPx+CAT)$ was significantly increased in DS and positively correlated with MDA ($p = 0.021$) and lipofuscin ($p = 0.059$). Of the antioxidant enzymes only CAT displayed a negative correlation with MDA ($p = 0.007$). The ratio R positively correlated with glutathione disulfide (GSSG) ($p = 0.048$) in DS, but no significant correlations for GSH or the ratio GSH/GSSG were found. We observed a negative correlation between

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the ratio R and TAS, resp. UA ($p = 0.048$ for TAS and $p = 0.039$ for UA) in DS, but not between R and vitamin E level. However, vitamin E displayed a positive correlation with TAS ($p = 0.048$). Similarly UA positively correlated with TAS ($p = 0.008$, for DS and $p = 0.045$ for controls). Our results may contribute to better understanding of DS pathogenesis, suggesting a beneficial role for adequate antioxidant therapy.

Key words: Down syndrome, antioxidant enzymes, glutathione, uric acid, vitamin E, malondialdehyde, lipofuscin.

Abbreviations: CAT, catalase; CV, coefficient of variation; DS, Down syndrome; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; Hb, hemoglobin; LMWA, low molecular weight antioxidant; MDA, malondialdehyde; SOD, Cu/Zn superoxide dismutase; TAS, total antioxidant status; UA, uric acid.

Introduction

It has been proposed that the pathogenesis of Down syndrome (DS) involves reactive oxygen species (ROS) (KEDZIORA & BARTOSZ, 1988). Persons with DS have an excess of Cu/Zn superoxide dismutase (SOD) enzyme activity in all cells by about 50 percent due to the presence of three copies of chromosome 21 instead of two. SOD catalyses the dismutation of superoxide to H_2O_2. Glutathione peroxidase (GPx) and catalase (CAT) than independently convert H_2O_2 to water (HALLIWELL & GUTTERIDGE, 1999; GAETA et al., 2002). SOD together with GPx and CAT form the main enzyme defense mechanism against harmful effects of ROS. The disequilibrium between the formation of hydrogen peroxide from superoxide in the dismutation reaction catalyzed by SOD and its degradation by GPx and CAT could represent a marker in development of oxidative stress noted in individuals with DS (MICHIELS et al., 1994; MUCHOVÁ et al., 2001).

Glutathione (GSH) is an important antioxidant, which takes part directly in elimination of toxic peroxides and aldehydes and indirectly in maintaining vitamins C and E in their reduced and functional forms (MEISTER, 1992; SAMIEC et al., 1998). Oxidative stress in cells exhausts GSH, producing its oxidized form GSSG. Measurement of glutathione disulfide and the ratio of GSH/GSSG are used as an index of the oxidative stress.

In our previous work (MUCHOVÁ et al., 1998; ŽITŇANOVÁ et al., 2004) we determined increased level of uric acid (UA) in DS. The function of UA is not quite clear. ŽITŇANOVÁ et al. (2004) assumed that UA in DS exhibits antioxidant activity, because increased level of non-enzymatic oxidative product of UA, allantoin, was proved.

The aim of this study was to investigate the relationship between the ratio of activities of antioxidant enzymes $R = \text{SOD}/(\text{GPx}+\text{CAT})$, and levels of non-enzymatic low molecular weight antioxidants (LMWA) (reduced and oxidized GSH, vitamin E, UA, total antioxidant status; TAS) as well as the concentrations of malondialdehyde (MDA) in erythrocytes, and lipofuscin in serum of persons with DS.

Material and methods

Subjects

We examined 44 individuals with Down syndrome, mean age 23.2 years and 26 healthy volunteers with a mean age of 23.3 years. All persons with DS had the cytogenetic diagnosis of trisomy 21. These patients are regularly monitored at the Child Development Center of Rhode Island Hospital (Providence, Rhode Island, USA). Individuals with DS who have additional chronic diseases, such as diabetes mellitus, and persons with acute respiratory illnesses were excluded from the study.

The studies were performed according to the Principles of the Helsinki Declaration. The project was approved by the Institutional Review Board of Rhode Island Hospital.

Methods

Isolation of erythrocytes. Heparinized (25 U/mL) fasting venous blood was washed three times with a 0.15 M NaCl solution. After centrifugation ($400 \times g$, 5 min) the erythrocytes were hemolysed by adding triple volume of distilled water. The Hb concentration in the hemolysate was measured using the Drabkin method.

Superoxide dismutase was determined by the modified spectrophotometric method of McCORD & FRIDOVICH (1969). 1U of SOD activity is defined as the enzyme activity causing 50% inhibition of reduction of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) under the assay conditions. For the quantification of SOD it was found, that 1U of SOD activity corresponded to 287 ng of standard SOD (Sigma) under the assay condition (GARAIOVÁ et

al., 2001). The activity of erythrocyte SOD is expressed in mg SOD/g Hb.

Glutathione peroxidase was determined using the RANSEL set (Randox, U.K.). Catalase was determined using the method by CAVAROCCHI et al. (1986). The activity of erythrocyte GPx and CAT was expressed as katal per gram of Hb.

Lipid peroxidation. Malondialdehyde in erythrocytes was determined using the reaction with thiobarbituric acid (UCHIYAMA & MIHARA, 1978). Amplification of peroxidation during the assay was prevented by the addition of the chain-breaking antioxidant butylated hydroxytoluene (10 μL of 2 mmol/L per 1 mL of blood) to the heparinised blood. The level of thiobarbituric acid reactive products was expressed as MDA (μmol/g Hb) using 1,1,3,3-tetraethoxypropane as standard.

Lipofuscin. Fluorescence determination of lipofuscin was based on the yellow autofluorescence of lipofuscin when excited by UV light (345 nm); this was measured at 430 nm using an AMICO-BOWMAN spectrofluorometer (ROUMEN et al., 1994). A quinine suphate solution in 0.1 M sulphuric acid and further diluted with phosphate buffer, was used to construct a standard curve. Lipofuscin concentration is expressed as arbitrary units (expressed as μmol/L) relative to the fluorescence of quinine sulphate.

Reduced glutathione was determined by the spectrophotometric method, using the BIOXYTECH GSH-400 kit (France), following deproteinization of blood with metaphosphoric acid. GSH content was expressed in μmol/g Hb.

Oxidized glutathione was determined using a modified method of VIDELA & JUNQUEIRA (1994), following deproteinization of blood with 0.5 M HClO$_4$. The GSSG content was expressed in μmol/g Hb.

Vitamin E was determined by the modified HPLC method (HESS et al., 1991) performed on a Beckmann chromatograph system equipped with 250 $\times$ 4.6 mm analytical column packed with Ultrasphere ODS 5 μm (Beckmann No. 235329, San Ramon, USA). Vitamin E fluorescence detection was performed at 298 nm/328 nm (excitation/emission). Quantification of vitamin E was performed by comparison of detected peak areas to those of external standards. Vitamin E concentration was calculated and expressed in μmol/L.

Uric acid was determined using kit and a photochemiluminescent method (PCL) from F.A.T. Berlin (POPOV & LEWIN, 1994). This method is based on an antioxidant-sensitive inhibition of photo-induced chemiluminescence accompanied by autooxidation of luminol. The concentration of UA is expressed in μmol/L of serum.

Total antioxidant status was determined using TAS kits (Randox, U.K.). The TAS concentration is expressed in mmol/L of serum. Trolox was used as a standard.

Statistical analysis
All results are presented as mean $\pm$ standard error (SEM) for the indicated number of observations and analyzed with the paired t Student test. Each test was repeated at least twice for every patient sample. Comparisons between DS and control group were performed using the Analysis of Variance (ANOVA). Correlations were expressed using the Pearson's product-moment correlation coefficient. A p-value of less than 0.05 was considered statistically significant. The quality parameters of the assays are lower than 5% for intraserial coefficient of variation (CV) and lower than 7% for interserial CV.

Results

The activities of antioxidant enzymes (SOD, GPx, CAT) in erythrocytes of persons with DS and the control group are shown in Table 1. Changes in the equilibrium between the formation of hydrogen peroxide from superoxide dismutation and its decomposition by other enzymes (GPx, CAT) in erythrocytes can be expressed by the ratio R = SOD/(GPx+CAT). This ratio was significantly different in persons with DS when compared with controls (0.014 $\pm$ 0.001 versus controls 0.010 $\pm$ 0.001; $p = 0.006$).

Plasma glutathione in reduced form (GSH) as a representative of low molecular weight antioxidant, as well as its oxidized form (GSSG) and the ratio GSH/GSSG were determined. There is a statistically significant decrease in the GSSG concentration of DS group (1.065 $\pm$ 0.030 μmol/g Hb versus controls 1.187 $\pm$ 0.032 μmol/g Hb; $p = 0.012$). Likewise, an insignificant decrease in the GSH concentration of DS persons was observed (6.894 $\pm$ 0.232 μmol/g Hb versus controls 7.555 $\pm$ 0.233 μmol/g Hb; $p = 0.064$). However, no statistically significant difference in the ratio of GSH/GSSG was found between the DS and control groups (6.587 $\pm$ 0.224 versus controls 6.515 $\pm$ 0.311; $p = 0.848$).

One of the defense mechanisms that protects against free radicals damage is non-enzymatic LMWA. The levels of TAS, vitamin E and UA were studied (Table 1). There is a significant decrease in levels of TAS (1.166 $\pm$ 0.022 mM versus controls 1.227 $\pm$ 0.021 mM; $p = 0.031$) and a significant increase in level of UA of DS individuals (364.117 $\pm$ 17.393 mM versus controls 293.522 $\pm$ 16.681 mM; $p = 0.007$).

The concentrations of lipid peroxidation products, erythrocyte MDA and serum lipofuscin are shown in Table 1. When MDA levels in erythrocytes of the DS and control groups were compared, there was a significant difference (1.582 $\pm$ 0.020 μmol/g Hb versus controls 1.504 $\pm$ 0.024 μmol/g Hb; $p = 0.019$). No statistically significant difference in lipofuscin levels between the DS and con-

Table 1. Clinical and biochemical characteristics of patients. [a]

Parameter	Down syndrome	Control	Significance
	Mean ± SEM (n)	Mean ± SEM (n)	p
A. Age and BMI			
Age (years)	23.208 ± 1.967 (44)	23.340 ± 2.978 (26)	NS
BMI (kg/m^2)	28.949 ± 1.308 (42)	N/A	
B. Activities of antioxidant enzymes in erythrocytes			
SOD (mg/g Hb)	1.139 ± 0.042 (42)	0.772 ± 0.034 (26)	< 0.001
GPx (μkat/g Hb)	0.657 ± 0.022 (43)	0.455 ± 0.027 (26)	< 0.001
CAT (μkat/g Hb)	87.190 ± 3.683 (41)	83.189 ± 4.735 (25)	NS
C. Levels of glutathione			
GSH (μmol/g Hb)	6.894 ± 0.232 (40)	7.555 ± 0.233 (26)	0.064
GSSG (μmol/g Hb)	1.065 ± 0.030 (40)	1.187 ± 0.032 (25)	0.012
GSH/GSSG	6.587 ± 0.224 (40)	6.515 ± 0.311 (25)	NS
D. Levels of low molecular weight antioxidant			
Vit. E (μmol/L)	21.147 ± 1.186 (38)	N/A	
Uric acid (μmol/L)	364.117 ± 17.393 (30)	293.522 ± 16.681 (25)	0.007
TAS (mmol/L)	1.166 ± 0.022 (42)	1.227 ± 0.021 (25)	0.031
E. Levels of oxidative degradation products			
MDA (μmol/gHb)	1.582 ± 0.020 (42)	1.504 ± 0.024 (24)	0.019
Lipofuscin (μmol/L)	11.718 ± 0.363 (34)	12.183 ± 0.595 (18)	NS
F. Ratio of antioxidant enzymes			
R (erythrocytes)	0.014 ± 0.001 (38)	0.010 ± 0.001 (24)	0.006

[a] 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 is the number of subjects per group; R = SOD/(GPx+CAT) (activities of enzymes in erythrocytes). N/A – not available.
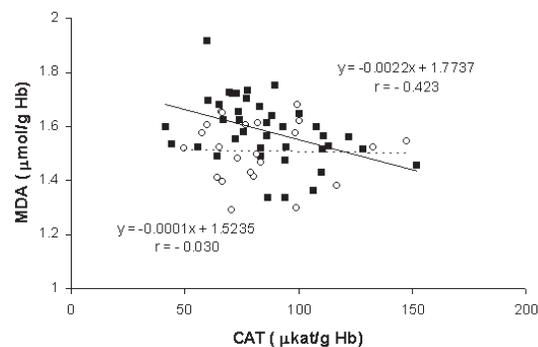

Fig. 1. Correlation between CAT activity and MDA level in erythrocytes of persons with DS (■, solid line; r = −0.423, p = 0.007, n = 39) and controls (○, dotted line; r = −0.030, p = 0.889, n = 23).
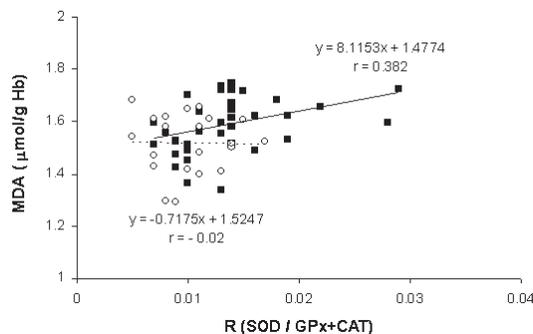

Fig. 2. Correlation between the ratio R = SOD/(GPx+CAT) (activities of enzymes) and MDA level in erythrocytes of persons with DS (■, solid line; r = 0.382, p = 0.021, n = 36) and controls (○, dotted line; r = −0.020, p = 0.929, n = 21).

trol groups was found (11.718 ± 0.363 μM versus controls 12.183 ± 0.595 μM; p = 0.665).

No significant correlations between the levels of lipid peroxidation products (MDA and lipofus-
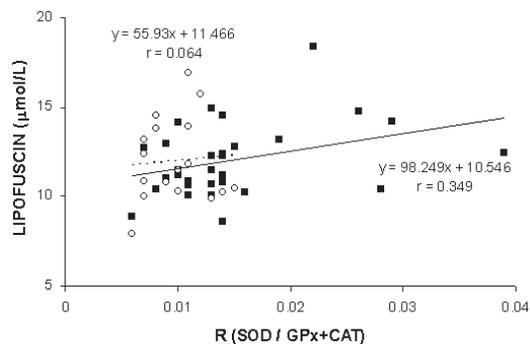
Fig. 3. Correlation between the ratio $R = \mathrm{SOD}/(\mathrm{GPx}+ \mathrm{CAT})$ (activities of enzymes) and lipofuscin in serum of person with DS (■, solid line; $r = 0.349$, $p = 0.059$, $n = 30$) and controls (○, dotted line; $r = 0.064$, $p = 0.806$, $n = 17$).
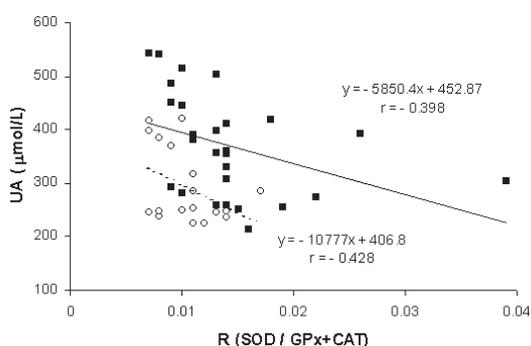

Fig. 5. Correlation between the ratio $R = \mathrm{SOD}/(\mathrm{GPx}+ \mathrm{CAT})$ (activities of enzymes) and UA in serum of person with DS (■, solid line; $r = -0.398$, $p = 0.039$, $n = 27$) and controls (○, dotted line; $r = -0.428$, $p = 0.076$, $n = 18$).
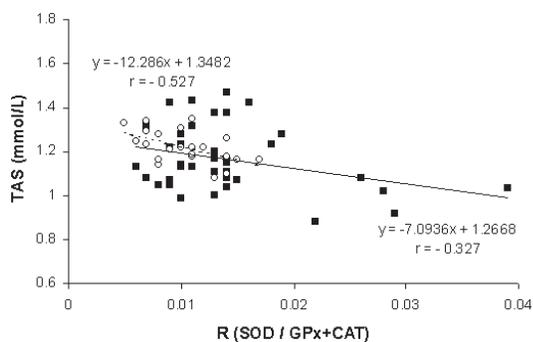

Fig. 4. Correlation between the ratio $R = \mathrm{SOD}/(\mathrm{GPx}+ \mathrm{CAT})$ (activities of enzymes) and TAS in serum of person with DS (■, solid line; $r = -0.327$, $p = 0.048$, $n = 37$) and controls (○, dotted line; $r = -0.527$, $p = 0.012$, $n = 22$).
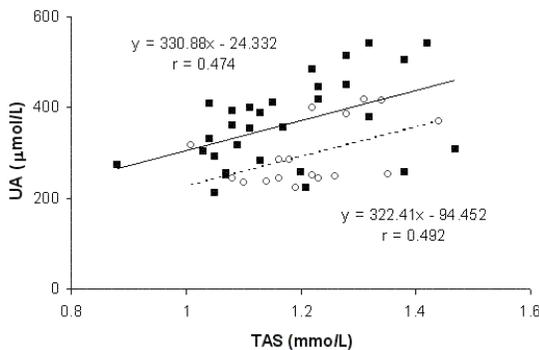

Fig. 6. Correlation between TAS and UA in serum of persons with DS (■, solid line; $r = 0.474$, $p = 0.008$, $n = 30$) and controls (○, dotted line; $r = 0.492$, $p = 0.045$, $n = 17$).

cin) and the individual antioxidant enzymes SOD and GR in erythrocytes were observed, except negative correlation of MDA levels with CAT activity in erythrocytes ($r = -0.423$, $p = 0.007$) for DS (Fig. 1).

The positive correlation between the ratio R and erythrocyte MDA for DS group ($r = 0.382$, $p = 0.021$) is shown in Figure 2 ($r = -0.020$, $p = 0.929$). Similar results were observed for serum lipofuscin with ratio R ($r = 0.348$, $p = 0.059$) (Fig. 3) and for GSSG with ratio R ($r = 0.341$, $p = 0.048$) in individuals with DS. No significant effect of GSH/GSSG ratio on R ratio, MDA or lipofuscin level was found.

In this study the correlations between the ratio R and LMWA, which are represented by TAS or individual antioxidants (vitamin E and UA), were monitored. The significant negative correlation between the ratio R and TAS for DS group as well as control group ($r = -0.327$, $p = 0.048$ for DS and $r = -0.527$, $p = 0.012$ for control) is shown in Figure 4. Similar negative correlation was observed between the ratio R and serum UA for DS group ($r = -0.398$, $p = 0.039$), although the correlation was insignificant for control group ($r = -0.428$, $p = 0.076$) (Fig. 5). The ratio R did not have a significant effect on the level of vitamin E in plasma of persons with DS either control group.

The relationship between TAS and individual LMWA was also studied. Figure 6 shows a significant positive correlation between TAS and UA

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levels for both groups ($r = 0.474$, $p = 0.008$ for DS and $r = 0.492$, $p = 0.045$ for control). Thus, significant positive correlation between TAS and vitamin E for DS group was noted ($r = 0.337$, $p = 0.048$).

No statistically significant difference was found in age composition of the persons with DS and control group (23.208 ± 1.967 years versus controls 23.340 ± 2.978; $p = 0.970$).

Discussion

Trisomic cells, though having increased SOD activity (KEDZIORA & BARTOSZ, 1988; DICKINSON & SINGH, 1993), are more sensitive to oxidative stress. In our previous study (MUCHOVÁ et al., 2001) in consent with MICHIELS et al. (1994) it was found that the sensitivity of cells to free radicals apparently depends on the equilibrium between the formation of hydrogen peroxide from superoxide in the dismutation reaction catalyzed by SOD and its degradation by GPx and CAT, rather than on the activities of individual antioxidant enzymes. In erythrocytes of persons with DS the ratio of SOD/(GPx+CAT) is higher than in the cells of controls (Table 1), which may result in an increased production of hydrogen peroxide in the cells of persons with DS. This finding may be supported by our unpublished results. An increased level of H_2O_2 in urine of persons with DS in comparison to the controls was observed (21.5 ± 2.3, $n = 23$ versus controls 14.3 ± 1.7, $n = 9$; $p = 0.046$). Results concerning the disequilibrium in antioxidant enzymes activities are in accordance with studies carried out on Slovakian persons with DS (MUCHOVÁ et al., 2001). The SOD/GPx ratio was also increased in fibroblasts of DS individuals (TEKSEN et al., 1998). In addition, BROOKSBANK & BALAZS (1984) reported an increase in SOD activity in cerebral cortex from DS fetuses not compensated by arising in GPx activity. Epidermal cells with overproduction of SOD are very sensitive to superoxide and hydrogen peroxide, whereas cells with overproduction of CAT are protected against effects of oxidants (AMSTAD et al., 1991). The protective effect of CAT was confirmed, as we observed lower MDA levels in erythrocytes with higher activity of CAT (Fig. 1).

We observed an interrelationship between the increased ratio $R = SOD/(GPx+CAT)$ and the oxidative stress markers, MDA (Fig. 2), lipofuscin (Fig. 3) and GSSG in individuals with DS. These results may support the theory of the contribution of antioxidant enzyme disequilibrium to oxidative stress in persons with DS due to limited

CAT activity and most likely to the insufficiently increased GPx activity. Moreover, the increased levels of the lipid oxidation product, erythrocyte MDA, found in this study, suggest that the balance of oxygen metabolism is compromised in persons with DS. However, we did not observe a difference in serum lipofuscin levels of DS persons (mean age 23.3 years) when compared to controls despite its significant increase with advancing age in subjects with DS ($r = 0.574$, $p = 0.0004$). This finding may support the hypothesis of premature aging in persons with Down syndrome over the age of 25 years, contrary to our previous results (MUCHOVÁ et al., 2001) found in Slovak individuals with DS (mean age 14.8 years) where lipofuscin levels did not correlate with age of DS patients.

Oxidative stress may reflect changes in the glutathione redox state in different tissues. In individuals with DS as well as in animal DS models controversial results in levels of GSH and GSSG were reported. No significant differences of GSH, likewise to our results in humans, and GSSG levels were found in Portuguese children with DS and their siblings (PINTO et al., 2002) and in individuals over 14 years old (CENGIZ et al., 2002). The decreased level of GSH was observed in mice with trisomy 16, a model of the human trisomy 21 (STABEL-BUROW et al., 1997). Our results concerning the individual levels of GSH, GSSG and ratio GSH/GSSG are not distinct. On the one hand in DS group a moderate decrease of GSH level in comparison to controls was found ($p = 0.064$) (Table 1). GSSG level in DS individuals was abruptly decreased, too, and the ratio GSH/GSSG was unaffected. On the other hand, the level of GSSG positively correlates with the ratio $R = SOD/(GPx+CAT)$.

These findings suggest that failure of the enzyme antioxidant system may lead to failure in metabolism of LMWA systems. Negative correlation of the ratio R and TAS (Fig. 4) or UA (Fig. 5) indicates exhaustion of low molecular weight hydrophilic antioxidants during oxidative stress initiated by an imbalance in activities of antioxidant enzymes, SOD versus GPx and CAT. Levels of vitamin E in DS persons did not show significant correlations with the ratio R. This may be due to the hydrophobic properties of vitamin E being contrary to hydrophilic enzyme antioxidant systems. UA (Fig. 6) as well as vitamin E in DS contribute to TAS, which follows from the positive correlation.

The role of UA in individuals with DS was discussed in our previous work (ŽITŇANOVÁ et al., 2004), where, in agreement with the present

study, as well as with the results of Nagyová et al. (2000), a significant increase in the level of UA was found in DS persons. The exact cause of the increased UA levels in individuals with DS is not known. The hyperuricemia could be result of elevated activities of erythrocyte adenosine deaminase and adenosine phosphoribosyltransferase (Becker, 1993) or glomerular dysfunction in DS (Nishida et al., 1979). Another possibility is the induced increase of UA level, as an important antioxidant of plasma, by oxidative stress. Increased levels of allantoin, as a non-enzymatic oxidative product of urate metabolism, were found, which indicates the antioxidant role of UA in persons with DS during increased oxidative stress (Žitňanová et al., 2004). The antioxidant function of UA is supported by our results presented in this paper, where UA level was found increased with decreased ratio $R =$ SOD/(GPx+CAT). From this follows that UA – as an antioxidant – is exhausted during oxidative stress. Moreover, UA may work indirectly as antioxidant through cooperation with GSH. If GSH functions as an antioxidant, then from electrochemical potentials for $GS^{\cdot}/GS^{-}$ (+0.85 V) and for urate radical/urate (+0.52 V) follows a regeneration ability of urate for glutathione radical (Buettner & Jurkiewicz, 1993; Ďuračková, 1998). This assumption is supported by our results, where the ratio GSH/GSSG in individuals with DS did not change compared to the controls (Table 1). This may indicate that improvement of GSH/GSSG ratio is of possible therapeutic value in treatment of individuals with DS. Schuchmann & Heinemann (2000) observed a reduced spontaneous neuronal death rate in diploid and Ts16 cultures and increased GSH level of up to 126% after adding of GSH precursors, cysteine and cystine and tocopherol to the culture medium. The treatment with N-acetyl cysteine has also showed the similar positive effect on levels of GSH (Neal et al., 2003).

In summary, the careful evaluation of parameters representing the oxidative stress in DS and their relationship to repair mechanisms may contribute to better understanding of DS pathogenesis, suggesting the beneficial role of adequate antioxidant therapy.

Acknowledgements

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