Redox Report
Communications in Free Radical Research

ISSN: 1351-0002 (Print) 1743-2928 (Online) Journal homepage: www.tandfonline.com/journals/yrer20

The effect of polyphenolic extract from pine bark, Pycnogenol® on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD)

Monika Dvořáková, Monika Sivoňová, Jana Trebatická, Igor Škodáček, Iveta Waczuliková, Jana Muchová & Zdeňka Ďuračková

To cite this article: Monika Dvořáková, Monika Sivoňová, Jana Trebatická, Igor Škodáček, Iveta Waczuliková, Jana Muchová & Zdeňka Ďuračková (2006) The effect of polyphenolic extract from pine bark, Pycnogenol® on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD), Redox Report, 11:4, 163-172, DOI: 10.1179/135100006X116664

To link to this article: https://doi.org/10.1179/135100006X116664

Published online: 19 Jul 2013.

Research article

The effect of polyphenolic extract from pine bark, Pycnogenol®, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD)

Monika Dvořáková[1], Monika Sivoňová[1], Jana Trebatická[2], Igor Škodáček[2], Iveta Waczuliková[3], Jana Muchová[1], Zdeňka Ďuračková[1]

[1]*Department of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic*
[2]*Department of Child Psychiatry, Faculty of Medicine, University Hospital, Bratislava, Slovak Republic*
[3]*Department of Nuclear Physics and Biophysics, Division of Biomedical Physics, Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovak Republic*

Attention deficit hyperactivity disorder (ADHD) belongs to the neurodevelopmental disorders characterized by impulsivity, distractibility and hyperactivity. In the pathogenesis of ADHD genetic and non-genetic factors play an important role. It is assumed that one of non-genetic factors should be oxidative stress. Pycnogenol®, an extract from the pine bark, consists of bioflavonoids, catechins, procyanidins and phenolic acids . Pycnogenol® acts as powerful antioxidant, chelating agent; it stimulates the activities of some enzymes, like SOD, eNOS, and exhibits other biological activities.
Aim: The aim of this randomized, double-blind, placebo-controlled trial was to investigate the influence of administered Pycnogenol® or placebo on the level of reduced (GSH) and oxidized (GSSG) glutathione in children suffering from ADHD and on total antioxidant status (TAS). This is the first investigation of the redox glutathione state in relation to ADHD.
Results: One month of Pycnogenol® administration (1 mg/kg body weight/day) caused a significant decrease in GSSG and a highly significant increase in GSH levels as well as improvement of GSH/GSSG ratio in comparison to a group of patients taking a placebo. TAS in children with ADHD was decreased in comparison with reference values. Pycnogenol® administration normalizes TAS of ADHD children.

Keywords: Oxidative stress, ADHD, Pycnogenol, glutathione, TAS

INTRODUCTION

Oxygen is fundamental for the production of energy (ATP) by all aerobic life forms. However, oxidative processes also generate highly reactive oxygen free radicals and their reactive metabolites in tissues. This is the reason for oxidative stress. Oxidative stress is defined as an imbalance in antioxidant and pro-oxidant levels to the benefit of pro-oxidants, which results in damage to biomolecules.[1] Oxidative stress is important in many diseases, *e.g.* atherosclerosis, neurodegeneration or ischemia-reperfusion states.[2] It is predicted that reactive oxygen species also play a role in biological stress[3] and in the etiology of many psychiatric diseases.[4]

Received 28 March 2006
Revised 1 July 2006
Accepted 12 July 2006

Correspondence to: Prof. Ing. Zdeňka Ďuračková PhD, Department of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Sasinkova 2, 811 08 Bratislava, Slovak Republic
Tel: +421 2 59357411; Fax: +421 2 59357557;
E-mail: zdenka.durackova@fmed.uniba.sk

Abbreviations: CHES, 2-(N-cyclohexamine)ethanesulphonic acid; ADHD, attention deficit hyperactivity disorder; FDNB, fluorodinitrobenzene; MDA, malondialdehyde; NEM, N–ethylmaleimide; GSSG, oxidized glutathione; PCA, perchloric acid; ROS; reactive oxygen species; GSH, reduced glutathione; SOD, superoxide dismutase; TAS, total antioxidant status

DOI 10.1179/135100006X116664

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental psychiatric disorders in children. Children with ADHD suffer from inattention, impulsivity and hyperactivity. According to a variety of epidemiological data, the incidence of ADHD in children and adolescents ranges from 3–5%. Boys are 2.5–9.0 times more likely to be diagnosed with ADHD than girls.[5] The molecular basis of ADHD is not yet clear; however, it is known that in its etiology, genetic as well as non-genetic factors play an important role. It is believed that ADHD arises from a complex interaction of environmental and biological factors, with strong evidence for a genetic component.[6] Non-genetic factors include some prenatal (fetal exposure to alcohol, drugs and tobacco) and birth complications, lead poisoning or head injuries.[7]

Studies of ADHD neurochemistry, neuro-imaging and genetics have supported the view that ADHD is a familial disease involving differences in monoamine regulation and frontal–striatal neural circuitry. Dopamine and noradrenaline are known to take part in the normal function of the prefrontal cortex.[8] In the pathophysiology of ADHD, damage to adrenaline, noradrenaline and dopamine metabolism occurs. These changes can modify attention, thinking and acting.[9] Damage in catecholamine's metabolism is considered as one of the possible sources of free radical formation. *In vitro*, catecholamines at higher concentrations in the presence of oxygen can be easily oxidized. Oxidation of catecholamines results in *o*–semiquinone formation. Under physiological conditions, semiquinone reacts with ascorbic acid, glutathione or other sulfhydryl groups to form stable thiol products. However, when semiquinone reacts with oxygen (*e.g.* in the brain), superoxide radical is formed, regenerating *o*–quinones. Generated *o*–quinones are susceptible to further metabolism to reactive semiquinone. In this reaction cycle, increasing amounts of superoxide radical and subsequently other reactive oxygen species are created.[10,11] *In vivo*, this can lead to oxidative stress and to neurodegenerative processes.[9,12]

During oxidative processes, H_2O_2 is formed. Under physiological conditions, H_2O_2 is inactivated by catalase or glutathione peroxidase using reduced glutathione (GSH) as co-factor. Toxicity of H_2O_2 is caused not only by its oxidant power, but also because of its reactivity with Fe^{2+} and other heavy metals ions in the Fenton-type reaction when a highly reactive hydroxyl radical ($^{\bullet}OH$) is formed.

It is assumed that oxidative stress plays a role in the pathophysiology of ADHD.[13] In recent years, antioxidant therapy of ADHD has been discussed[14] as a supplement to classical treatment by psychostimulants, antidepressants, neuroleptics and mood stabilisators. However, different side-effects of these psychomedicaments were found.[15,16]

Several reports have suggested a beneficial effect of Pycnogenol® on ADHD symptoms in children. First case reports concerning positive effects following treatment of children with ADHD with Pycnogenol® were collected by Passwater (1998),[17] Heimann (1999),[18] and Masao (2000).[19] An attempt to demonstrate reduction of ADHD symptoms in adults failed in a double-blind, placebo-controlled, comparative study with 24 adults.[20] We also found a significant improvement of ADHD symptoms in children after 1 month of Pycnogenol® 1 mg/kg/day administration.[14]

Pycnogenol® (Horphag Res. Ltd, Geneva, Switzerland) is a standardized extract from the French pine (*Pinus pinaster*) bark-concentrate of polyphenols consisting of procyanidins, catechin, taxifoline and phenolic acids.[21] It acts as a strong scavenger of free radicals *in vitro*[21] and it stimulates activities of antioxidant enzyme (Cu/Zn superoxide dismutase).[22] We have shown[13] that Pycnogenol® lowers oxidative damage to DNA (expressed as 8-oxoG/10^6 G) of children suffering from ADHD. This is an important finding as a significant increase in DNA damage was found in ADHD children compared to a group of healthy children.[13]

Pycnogenol® increases vasodilatation by stimulating the activity of endothelial NO-synthase.[23] Except for the pathological effects of higher concentrations of nitric oxide (neurodegeneration or neuro-inflammation), at physiological concentrations NO plays a crucial role in the brain (*e.g.* neuromodulation, neurotransmission and synaptic plasticity).

Pycnogenol® influences glutathione metabolism through elevation of glutathione peroxidase and glutathione reductase activities.[24] In addition, Pycnogenol® can scavenge produced free radicals and so spares GSH; this can indirectly lead to the elevation of GSH levels.[25]

Glutathione is one of the most abundant intracellular antioxidants. GSH (γ-glutamyl-cysteinyl-glycine) plays a key role in the protection of proteins, lipids and nucleic acids against free radical damage. There is also a direct correlation between the speed of aging and the reduction of GSH concentrations in intracellular fluids.[26,27]

GSH has potent electron-donating capacity, as indicated by the high negative redox potential of the GSH/GSSG redox couple ($E'_0 = -0.33$ V).[28] This renders GSH an antioxidant capacity and it is a convenient co-factor for enzymatic reactions. The reducing power of GSH is related to its free radical scavenging, electron-donating and sulfhydryl-donating capacity.

In addition, glutathione regulates protein activity via GSH transferases and thioredoxin[29] and it is also the source of amino acids for protein synthesis.[30] A deficit of GSH would lead to degenerative processes at dopaminergic terminals resulting in loss of connectivity.[9]

Healthy brain cells possess high concentrations of both enzymatic (Cu/Zn- and Mn-superoxide dismutases,

GSH-peroxidase) and non-enzymatic antioxidants (glutathione, vitamins C and E).[31] Under physiological conditions, cells are able to cope with the toxicity of ROS with the help of an antioxidative pool. However, free radicals can cause peroxidation and microlesions of the membrane in neurons. This is known to be a primary cause of many degenerative diseases (schizophrenia, Alzheimer's disease, Parkinson's disease).

Determination and evaluation of both GSH and GSSG and their ratio in blood has been considered essential as an index of several physiological and pathological situations. A decreased level of the reduced form of glutathione contributes to the disequilibration in oxidant/antioxidant balance in the organism following increased oxidative stress.

Presently, much attention is being devoted to supplementation with natural polyphenols and flavonoids showing antioxidant properties,[32,33] like Pycnogenol®.[23]

In our pilot study,[34,35] we found increased levels of malondialdehyde (MDA) and decreased total antioxidant status (TAS) in ADHD children in comparison to a control group. In a recent paper,[13] we found increased levels of 8-oxodeoxyguanosine (8-oxodG) in ADHD children. Therefore, we suggest an increased oxidative stress in these children.

The aim of this work was to determine the antioxidant and redox states of ADHD patients represented by reduced and oxidized glutathione and to determine how Pycnogenol® affects these markers.

Patients and Methods

Patients

A total of 43 out-patients (34 boys and 9 girls; aged 6–14 years) with ADHD, treated at the Child Psychiatric Clinic of the Children's University Hospital, were enrolled in a randomized, double-blind and placebo-controlled study. Patients were randomized to receive either Pycnogenol® or placebo.

Inclusion criteria

Inclusion criteria included: early onset of ADHD (by 6–7 years), chronicity, disorders of cognitive function (inattention, distractibility), difficulty persisting with any one task, difficulty in selective process to information, disturbance of the executive functions (production, sequention and realization of plans), disturbance of motivation, effort and fortitude, visuospacial and memory disturbance.

Exclusion criteria

Exclusion criteria included: situational hyperactivity, pervasive developmental disorders, schizophrenia, other psychotic disorders (mood, anxiety), personality disorder (as unsocial behavior), personality change due to a general medical condition, mental retardation, understimulating environments, conduct disorder, tics, chorea and other dyskinesias. Patients with acute inflammatory diseases, renal and cardiovascular disorders and diabetes were excluded from this study, too.

The Ethical Committee of the Children's University Hospital approved the study. Parents gave written consent for participation of their children in the study.

Medication

Children were supplemented with either Pycnogenol® (1 mg/kg body weight/day) or placebo (with identical shape and appearance and same number of pills/day as in the case of Pycnogenol®) for 1 month. The placebo contained lactose (58 mg) and cellulose (65 mg). Both, Pycnogenol® and placebo tablets were produced by Drug Research Institute, Modra, Slovakia.

Selection into the Pycnogenol® or placebo group was carefully randomized. The ratio of these groups was 2.5:1. The sample size was estimated assuming the power of 80% (β = 20%), the type one error (α) of 5% and the number of controls per subject, 0.4. The recommended number of patients was pre-calculated as 41 for drug investigation and 16 subjects for placebo. We included in the study 44 and 17 patients, respectively. Stat Direct® v.2.3.7 was used for the unpaired random allocation to intervention or control group and for the sample size estimation.

Patients had a standard diet and were not treated with other psychotropic drugs or with vitamins E and C during the study period.

Patient treatment

Patients were studied at the beginning of the trial before Pycnogenol®/placebo administration (0), after 1 month of Pycnogenol®/placebo administration (1), and 1 month after termination of treatment (wash-out period) (2).

Venous blood samples were taken at all investigated periods into commercial tubes with sodium citrate as an anticoagulant. Whole blood was used for determination of total and oxidized glutathione. The rest of blood samples was centrifuged and plasma was aliquoted, shock frozen and stored at –80°C until further analysis.

Chemicals

Perchloric acid (PCA), N–ethylmaleimide (NEM), bapthophenanthrolinedisulfonic acid (BPDS), iodoacetic acid, 2-(N-cyclohexamine)ethanesulphonic acid (CHES), oxidized glutathione, reduced glutathione, metacresol

purple, fluorodinitrobenzene (FDNB), sodium acetate, and acetic acid were purchased from Sigma-Aldrich Chemical (Taufkirchen, Germany). Potassium hydroxide (KOH) was obtained from Lachema (Brno, Czech Republic). Ethanol and methanol were purchased from Merck (Darmstadt, Germany).

Reagents

Solution 1 contained 12% PCA in water, 1.2 mM BPDS in water, 40 mM NEM in water. Solution 2 contained 6% PCA in water, 1 mM BPDS in water. Solution 3 contained 3 M KOH in water, 0.3 M CHES in water. This

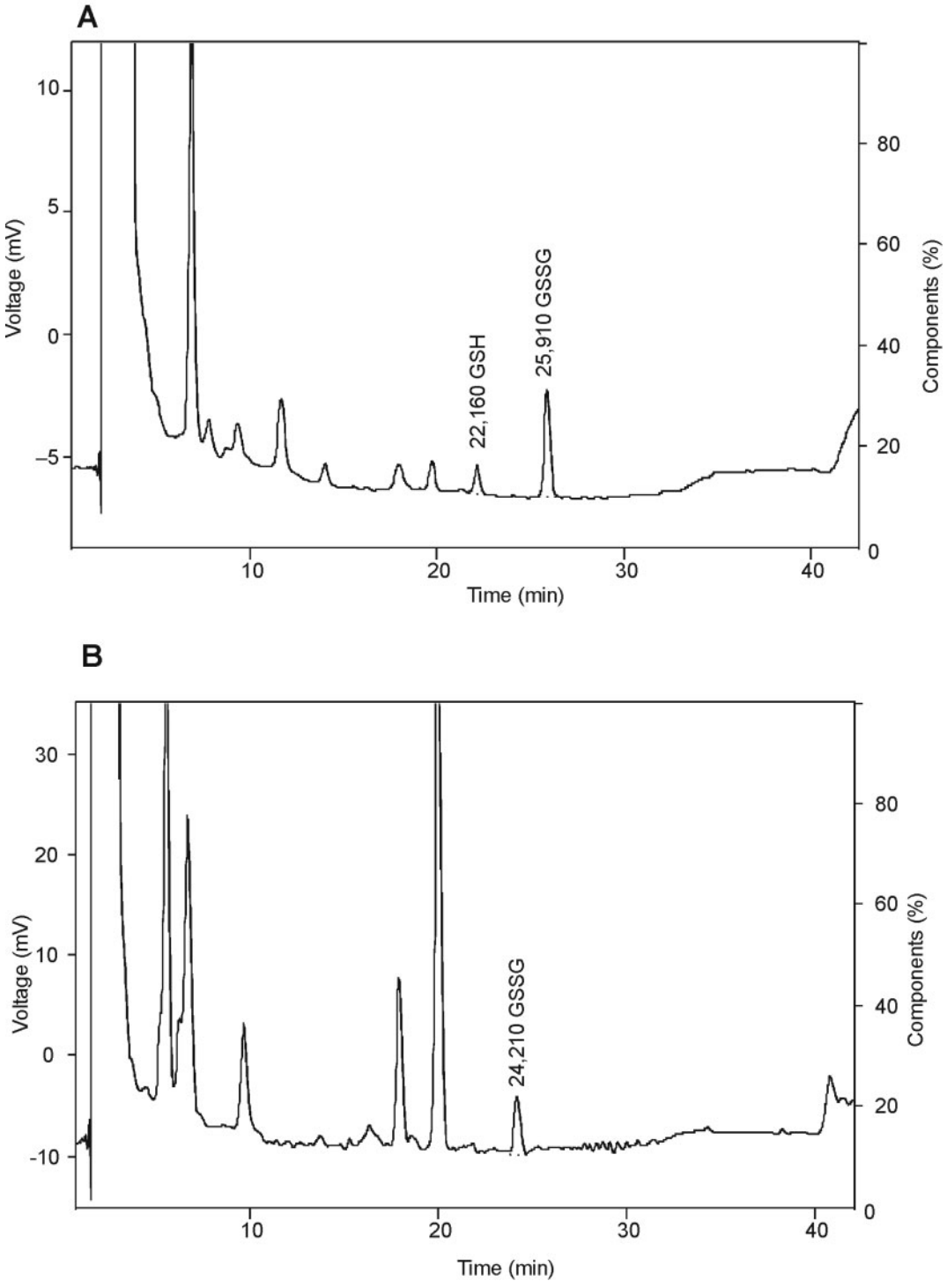

Fig. 1. Chromatography record of HPLC determination of oxidized (A) and total (B) glutathione. HPLC conditions: 20 × 0.46 cm column Allsphere amino (Alltech); mobile phases A and B are mixed together according to following gradient: t_0–t_8, 80:20 (A:B) (%); t_8–t_{30}, 1:99 (A:B) (%); t_{30}–t_{38}, 1:99 (A:B) (%); t_{38}–t_{42}, 80:20 (A:B) (%); t_{42}–t_{49}, 80:20 (A:B) (%); flow rate of 1 ml/min; injection 70 µl; detection on UV detector (DeltaChrom UVD200) at 375 nm.

buffer keeps pH between 8.5–9. pH indicator 1 consisted of 0.2 mM metacresol purple in water: a change to purple color occurs at pH 9. pH indicator 2 was 10 M iodoacetic acid in 0.2 mM metacresol purple. The detecting reagent was 1% FDNB in 96% ethanol. The acetic acid solution contained 3.3 M sodium acetate, 11.025 M acetic acid in water. Mobile phase A was 80% methanol in water. Mobile phase B was a 20% solution of acetic acid in 80% mobile phase A. Both mobile phases were filtered and de-aerated.

Analytical methods

Glutathione determination

Under physiological conditions, the GSH concentration is much higher than the GSSG concentration. GSH very easily undergoes auto-oxidation to GSSG, especially in the presence of metals. To impede its oxidation, substances blocking thiol groups are used (N-ethylmaleimide, iodoacetic acid). Concentrations of total glutathione and GSSG in whole blood were determined separately by gradient HPLC according to a modified method of Reed *et al*.[36] and detected at 375 nm. Afterwards, the GSH concentration was determined indirectly from values of GSSG and total glutathione:

$$[GSH] = [\text{total glutathione}] - (2 \times [GSSG]) \quad \text{Eq. 1}$$

HPLC conditions for both, total and oxidized glutathione

For the separation of total and oxidized glutathione, an aminocolumn Allsphere amino (Alltech, Deerfield, IL, USA) 20 × 0.46 cm was used. The size of filling mass was 5 μm. A 20 μl aliquot of prepared sample (see below) was directly injected onto the column. The flow rate of the mobile phase was 1 ml/min and glutathione was detected at 375 nm with a UV detector (DeltaChrom UVD 200, Watrex, Czech Republic).

A multistep gradient was used with a starting ratio 80:20 (A:B) and remained isocratic for 8 min. At this time, the composition was changed linearly to A:B (1:99) in 30 min. This state was isocratic until 38 min then changed to A:B (80:20) in 42 min. The separation finished in 49 min at A:B (80:20) as shown in Figure 1.

Total glutathione (TG)

For constructing a calibration curve, standards at concentrations of 6.25–100 μmol/l were prepared. Standards were then adjusted in the same way as samples – 800 μl of solution 2 was added to 200 μl of blood sample/standard. Such modified samples were stored at –20°C until use.

Before measurement, treated samples were centrifuged at 4°C, for 6 min at 14,000 *g*. Then 20 μl of pH indicator 2 was added to 200 μl of supernatant and the pH was adjusted to 9–9.5 with solution 3. After 30 min incubation at room temperature in the dark, 400 μl of FDNB was added to the sample/standard. Mixed samples/standards were incubated for 24 h at 4°C. The amount of total glutathione was measured by gradient HPLC at 375 nm.

Total glutathione was measured as the sum of GSH and GSSG, which accrued by spontaneous oxidation of GSH. Total glutathione was calculated from:

$$[TG] = ([GSH] + \{2 \times [GSSG]\}) \times 5 \text{ (dilution)} \quad \text{Eq. 2}$$

where: [GSH] = concentration of reduced glutathione as a part of total glutathione and [GSSG] = concentration of oxidized glutathione as a part of total glutathione. The concentration of total glutathione is expressed as μmol/l.

Oxidized glutathione (GSSG)

For constructing a calibration curve, standards at concentrations of 6.25–100 μmol/l were prepared. Standards were then adjusted in the same way as samples – 800 μl of solution 1 were added to 200 μl of blood sample/standard. NEM binds to the –SH groups of reduced glutathione to prevent its oxidation. Such modified samples were stored at –20°C. Before analysis, modified samples were centrifuged at 4°C, for 6 min at 14,000 *g*. Then, 20 μl of pH indicator 1 was added to 200 μl of supernatant and the pH was adjusted to 9–9.5 with solution 3. Prepared samples/standards were centrifuged at 4°C, for 15 min at 15,000 *g* and 50 μl of FDNB was added to 25 μl of the supernatant. Samples/standards were incubated for 45 min at room temperature in the dark. Derivatized samples/standards were dried under vacuum and stored at –20°C in the dark until HPLC analysis. Samples/standards processed this way were stable for several weeks. Before loading on the column, the sample was dissolved in 200 μl of mobile phase A. The amount of oxidized glutathione was measured by gradient HPLC at 375 nm. The concentration of GSSG was calculated from:

$$[GSSG] = (P \times 2 \text{ (dilution)}) \quad \text{Eq. 3}$$

where: P = area of the peak of the oxidized glutathione; and [GSSG] = concentration of the oxidized glutathione. The concentration of oxidized glutathione is expressed as μmol/l.

Total antioxidant status

Total antioxidant status (TAS) in plasma was analysed by standard biochemical procedures using an Hitachi 911 automatic analyser with a Randox kit (UK). The TAS concentration is expressed in mmol/l of plasma using of Trolox as a standard.

Basic biochemical parameters

Basic biochemical parameters (bilirubin, glucose, γ-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, uric acid and lipid profile) were analysed in plasma by standard biochemical procedures using an Hitachi 911 automatic analyser and Roche kits (Switzerland).

Statistical analysis

Descriptive statistics were obtained for all variables using mean ± SEM for normally distributed continuous variables, or medians and 25th and 75th interquartile ranges (IQRs) for data showing departures from normality (according to Shapiro-Wilk's test). Categorical variables were described using frequencies and proportions. Due to considerable intersubject variability in the monitored parameters and the unequal number of subjects in the two groups, the baseline values differed, thus making impossible any decision on the effect of the treatment. Therefore, we further worked with differences of glutathione concentration levels between time 0 (the beginning of therapy with Pycnogenol®), time 1 (a month later, at the end of the therapy), as well as between time 0 and time 2 (2 months later, including 4 weeks of wash-out period). Standard Student's *t*-test for the comparison of raw data showed no departures from normality and the non-parametric Mann-Whitney U-test for differences was used as the inference test. The test for two independent proportions was used to evaluate qualitatively the dissimilarity of changes observed in subjects following treatment with placebo or Pycnogenol®. The associations between variables were analyzed with Pearson's correlation coefficients from the models of simple linear regression.

Outlying observations were censored using the typical tests for outliers (Dixon's Q-test and Grubbs' test) and smoothed by a process referred to as 'windsorizing'. In windsorizing, the extreme values (in our case just one value) are not eliminated from the data set but replaced by the value of the cut-off criterion.[37] Windsorizing is a compromise between the two goals of eliminating the strong influence of extreme values on the mean while at the same time utilizing all of the information in a data set.

For statistical analysis, we employed the statistical program StatsDirect® v.2.3.7 (StatsDirect Sales, Sale, UK). Graphical representation of data was made using Excel 2000 (Microsoft Co.).

RESULTS

Before the trial, all values of biochemical parameters (bilirubin, glucose, γ-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, uric acid and lipid profile) were in the physiological range for both groups. None of these parameters changed beyond the normal range of physiological values after 1 month of Pycnogenol® or placebo administration.

At the beginning of the study, the level of GSSG for the Pycnogenol® group was 4.60 ± 0.09 μmol/l. One month of Pycnogenol® administration caused a significant decrease in the level of GSSG by 22.03% (3.58 ± 0.51 μmol/l; $P = 0.013$). In the placebo group, no significant change was observed. After the wash-out period, the level of GSSG in the Pycnogenol® group increased again (Fig. 2).

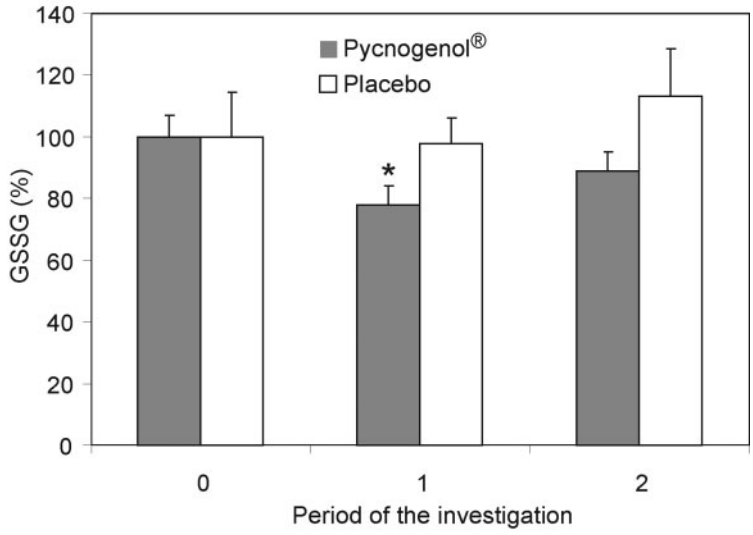

Fig. 2. Oxidized glutathione level in patients suffering from ADHD after Pycnogenol® (filled bar) ($n = 28$) and placebo (empty bar) ($n = 14$) administration. The level of GSSG at the beginning of the study was taken as 100%. Values represent mean in percentage ± SEM. 0, examination before the trial; 1, one month after Pycnogenol® or placebo administration; 2, one month after termination of administration (wash-out period). *Denotes significance between examination 0 and 1 ($P < 0.05$).

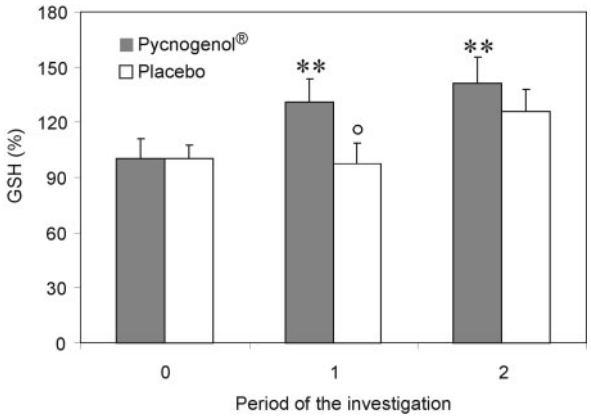

Fig. 3. Reduced glutathione level in patients suffering from ADHD after Pycnogenol® (filled bar) (n = 28) and placebo (empty bar) (n = 15) administration. The level of GSH at the beginning of the study was taken as 100%. Values represent mean in percentage ± SEM. 0, examination before the trial; 1, one month after Pycnogenol® or placebo administration; 2, one month after termination of administration (wash-out period). **Significance between examination 0 and 1 after Pycnogenol® administration (P < 0.01); °significance in examination 1 between Pycnogenol® and placebo (P < 0.05).

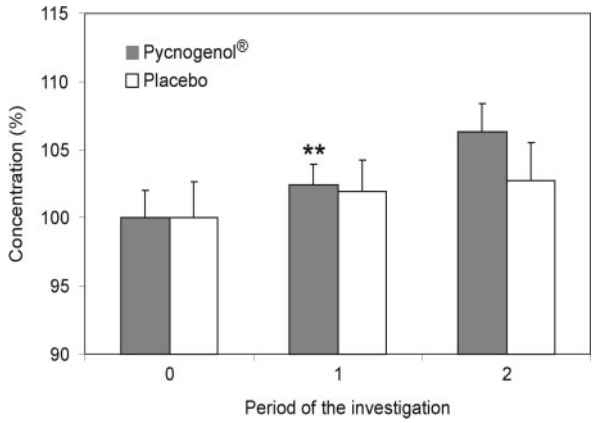

Fig. 4. TAS in patients suffering from ADHD after Pycnogenol® (filled bar) (n = 42) and placebo (empty bar) (n = 17) administration. TAS at the beginning of the study was taken as 100%. Values represent mean in percentage ± SEM. 0, examination before the trial; 1, one month after Pycnogenol® or placebo administration; 2, one month after termination of administration (wash-out period). **Significance 0/2 after Pycnogenol® administration (P < 0.01).

The level of GSH in the Pycnogenol® group at the beginning of the study was 102.89 ± 19.08 μmol/l (Fig. 3). A highly significant increase of GSH level by 26.8% was determined in the period of the investigation 1 in comparison to period 0 (130.44 ± 7.94 μmol/l; P = 0.0054) and this increase (36.4%) persisted also in the period of the investigation 2 (140.38 ± 60.74 μmol/l; P = 0.007) in patients taking Pycnogenol®. The level of GSH in patients taking placebo was not significantly changed.

TAS in children with ADHD is decreased (1.02 mmol/l) in comparison to reference values of 1.1–1.7 mmol/l. After 1 month of Pycnogenol® administration, TAS values increased slightly (1.05 ± 0.016 mmol/l); however, this elevation was not statistically significant in comparison to TAS level at the period of investigation 0. A statistically significant difference was recorded after the wash-out period (1.09 ± 0.02 mmol/l; P = 0.002). Placebo administration had no significant effect on TAS. TAS values ± SEM (in percentage) are shown in Figure 4.

There was also a negative correlation between measurement of TAS and concentration of oxidized glutathione after Pycnogenol® administration (n = 26, y = –4.457x + 9.0048, where y = GSSG concentration, x = TAS level, r = –0.3882, P < 0.05) as shown in Figure 5.

A positive correlation between inattention score and TAS normalizing is represented in depicted correlation (n = 33, y = –5.612x + 3.662 where y = inattention, x = TAS

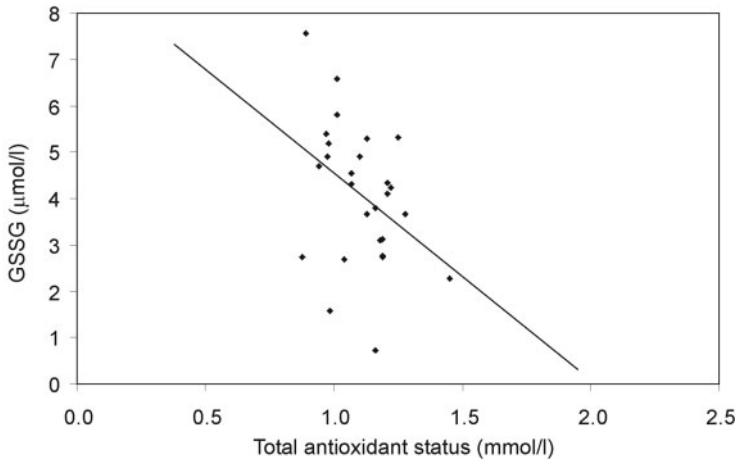

Fig. 5. Correlation between total antioxidant status and oxidized glutathione level (n = 26, y = -4.457x + 9.0048, r = -0.3882; P < 0.05).

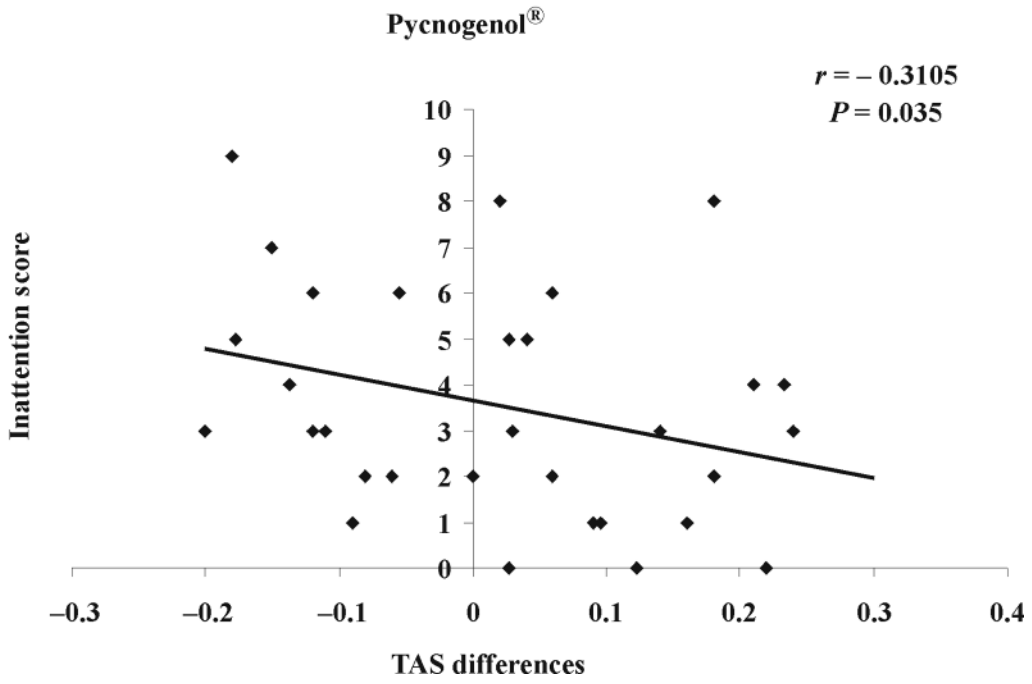

Fig. 6. Correlations between inattention score[13] in period 1 and TAS differences between periods 1 and 0 in Pycnogenol® group (n = 33, y = –5.612x + 3.662 where y = inattention, x = TAS level, P = 0,035). Period 0, examination before the trial; period 1, one month after Pycnogenol®/placebo administration.

level, P = 0.035) as shown in Figure 6. In the placebo group, no correlation was found.

Discussion

Imbalance in the levels of pro-oxidants and antioxidants to the benefit of pro-oxidants leads to elevated oxidative stress. One possible mechanism to protect the cell against free radical attack is to increase its antioxidant pool.

Measurement of the GSH/GSSG ratio has been suggested as a clinical marker in disorders in which oxidative stress plays a role. GSH is also oxidized to GSSG in ageing, which is a reflection of the accumulation of oxidative stress by the organism.[31] A decreased GSH/GSSG ratio is associated with tumor progression and many chronic diseases (gastrointestinal, cardiovascular, musculoskeletal).[38]

We calculated the GSH/GSSG ratio in patients with ADHD at the beginning of the trial as 35.93. After Pycnogenol® treatment, the GSH/GSSG ratio rose to 52.26 (P = 0.05). After a wash-out period, the ratio decreased again to 42.45. In a placebo group, the ratio GSH/GSSG was unchanged.

The concentration of GSH is also important in neurodegenerative disorders. Glutathione and free radicals have been recognized as playing a significant role in the development and progression of many neurodegenerative disorders. The brain is particularly susceptible to free radical attack, because it generates more free radical by-products per gram of tissue than any other organ. Glutathione, as the brain's important antioxidant, protects against this; for example, a significant decrease in the level of GSH (by 27%) was observed in the cerebrospinal fluid of drug-free schizophrenic patients.[9,12,39] The results of Castagne *et al.*[40] suggest that low brain glutathione and ascorbic acid levels associated with the dopaminergic system actively participate in the development of some cognitive deficits affecting patients with schizophrenia.

The results of Myhrstadt *et al.*[41] and Carlsen *et al.*[42] added regulation of GSH concentration to the list of diseases prevented by the effects of polyphenols.

Whereas in ADHD disease damage to catecholamine metabolism in the regulation of noradrenaline and dopamine release and uptake is predicted, damage to glutathione metabolism could share in cognitive deficit in patients suffering from ADHD. But a positive correlation between GSSG and adrenaline (n = 25, y = 1.3317x + 1.9984 where y = adrenaline level, x = GSSG level; P = 0.007) and GSSG and noradrenaline (n = 25, y = 2.9185x + 8.224 where y = noradrenaline, x = GSSG level; P = 0.003) has also been found. No correlation has been proven 1 month after Pycnogenol® administration (Dvořáková *et al.*, unpublished results).

Total antioxidant status (TAS) in children with ADHD is slightly decreased when compared to physiological values of healthy individuals. Pycnogenol® administration caused a slight, but insignificant, elevation of the TAS. It was interesting to note that improvement of the antioxidant status persisted after the wash-out period with a significant increase compared to period 0. In spite of the fact that Pycnogenol® exerts a marked antioxidant activity *in vitro* in a hydrophilic as well as a lipophilic environment,[43] *in vivo* (plasma) only a mild, non-significant increase of antioxidant capacity was found after 1 month of Pycnogenol® administration to children with ADHD (1 mg/kg/day). From the dose of administered Pycnogenol® (1 mg/kg body weight), assuming an average molecular weight of ~300 for the individual polyphenols present in Pycnogenol® and about a 30% absorption of polyphenols in the gastrointestinal tract, the mean concentrations of polyphenols in blood reaches about 10 μM. Children have higher concentrations of other antioxidants in blood (*e.g.* ascorbic acid, around 50 μM; tocopherols, 30 μM; and uric acid, 200–390 μM) than the calculated Pycnogenol® contribution. From this it follows that, under our conditions, the direct Pycnogenol® contribution to antioxidant capacity of plasma is not significant when compared to the relative higher levels of other plasma antioxidants.

We have shown a positive influence of Pycnogenol® on ADHD symptoms.[14] The relationship between inattention and TAS normalizing has also been confirmed (Fig. 6).[13] From this it follows that adequate intake of antioxidants (*e.g.* from food) may also have a positive influence on mental disorders like ADHD.

Similarly, a relationship between hyperactivity symptoms and noradrenaline (n = 37, y = 0.1678x + 6.4039 where y = hyperactivity score, x = noradrenaline level; $P = 0.031$) and hyperactivity and dopamine (n = 38, y = 0.0228x + 5.5812 where y = hyperactivity score, x = dopamine level; $P = 0.05$) levels has been found (Dvořáková *et al*., unpublished results).

However, in addition to the direct antioxidant activity, Pycnogenol® has the ability to stimulate activity of antioxidant enzymes such as SOD,[22] through both up-regulation of Cu/Zn-SOD protein expression[44] and increasing its activity.[45] Stimulation of SOD expression might persist even after discontinuation of polyphenol administration. Even though SOD is not the main plasma antioxidant enzyme, its elevated activity in cells may save low-molecular weight antioxidants, leading to the increase in their activity in plasma, which persists after termination of Pycnogenol® administration. Pycnogenol® also indirectly regenerates other antioxidant systems through increasing glutathione reductase activity.[22] In this way, regenerated glutathione (GSH) can also contribute to, and increase, TAS. This is also shown in our results, where significantly elevated levels of both TAS and GSH persisted after the wash-out period in comparison to period 0.

Conclusions

One month of Pycnogenol® administration to ADHD children normalises total antioxidant status and improves the redox state of the organism through a significant decrease of GSSG levels and a highly significant increase of GSH levels in comparison to a group taking placebo.

Acknowledgements

This study was supported by a grant from Horphag Res. Ltd, partly by VEGA grant No. 1/1157/04 and grant VV MVTS 03/LF from the Ministry of Education of the Slovak Republic, by Drug Research Institute, Modra, Slovak Republic and by Mind & Health, Civil Association. The authors wish to thank Assoc. Prof. P. Blazicek (Bratislava, Slovak Republic) for the biochemical analyses, Prof. Dr P. Rohdewald (University of Münster, Germany) for his helpful comments and Mrs L. Chandogová and L. Miková for their technical assistance.

References

1. Sies H. (ed) *Oxidative Stress: Oxidants and Prooxidants*. London: Academic Press, 1999.
2. Ďuračková Z. *Vol'né radikály a antioxidanty v medicine II*. Slovak Academic Press, 1999; 1–315.
3. Sivoňová M, Žitňanová I, Hlinčiková L, Škodácek I, Trebatická J, Ďuračková Z. Oxidative stress in university students during examinations. *Stress* 2004; **7**: 183–188.
4. Sirota P, Gavriely R, Wolach B. Overproduction of neutrophil radical oxygen species correlates with negative symptoms in schizophrenic patients: parallel studies on neutrophil chemotaxis, superoxide production and bactericidal activity. *Psychiatry Res* 2003; **121**: 123–132.
5. Barkley RA. *Attention Deficit Hyperactivity Disorder: A Handbook for Diagnosis and Treatment*. New York: Guilford Press, 1990.
6. Kaminester DD. Attention deficit hyperactivity disorder and methylphenidate: when society misunderstands medicine. *J Med* 1997; **3**: 105–114.
7. Anastopoulos AD, Shelton TL. *Assessing Attention – Deficit/ Hyperactivity Disorder*. New York: Kluwer/Plenum, 2001; 1–349.
8. Gillberg C, Gillberg IC, Rasmussen P *et al*. Co-existing disorders in ADHD – implications for diagnosis and intervention. *Eur Child Adolesc Psychiatry* 2004; **13**: 180–192.
9. Grima G, Benz B, Parpura V, Cuénod M, Do KQ. Dopamine – induced oxidative stress in neurons with glutathione deficit: implication for schizophrenia, *Schizophr Res* 2003; **62**: 213–224.
10. Baez S, Segura-Aguilar J, Widersten M, Johansson AS, Mannervik B. Glutathione transferases catalyse the detoxication of oxidized metabolites (*o*-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular

processes. *Biochem J* 1997; **324**: 25–28.
11. Smythies J. Redox aspects of signaling by catecholamines and their metabolites. *Antioxid Redox Signal* 2000; **2**: 575–83.
12. Do KQ, Trabesinger AH, Kirsten-Kruger M *et al*. Schizophrenia: glutathione deficit in cerebrospinal and prefrontal cortex *in vivo*. *Eur J Neurosci* 2000; **12**: 3721–3728.
13. Chovanová Z, Muchová J, Sivoňová M *et al*. Effect of polyphenolic extract, Pycnogenol®, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. *Free Radic Res* 2006; In press.
14. Trebatická J, Kopasová S, Hradečná Z *et al*. Treatment of ADHD with French maritime pine bark extract, Pycnogenol®. *Eur Child Adolesc Psychiatry* 2006; In press.
15. Simpson D, Plosker GL. Atomoxetine: a review of its use in adults with attention deficit hyperactivity disorder. *Drugs* 2004; **64**: 205–222.
16. Jeng W, Wong AW, Ting-A-Kee R, Wells PG. Methamphetamine – enhanced embryonic oxidative DNA damage and neurodevelopmental deficits. *Free Radic Biol Med* 2005; **39**: 317–326.
17. Passwater RA. *All about Pycnogenol®*. New York: Avery, 1998.
18. Heimann S. Pycnogenol® for ADHD? *J Am Child Adolesc Psychiatry* 1999; **38**: 357–358.
19. Masao H. Pycnogenol®'s therapeutic effect in improving ADHD symptoms in children. *Mainichi Shimbun*, 21 October 2000.
20. Tenenbaum S, Paull JC, Sparrow EC, Dodd DK, Green L. An experimental comparison of Pycnogenol® and methylphenidate in adults with attention-deficit/hyperactivity disorder (ADHD). *J Atten Disord* 2002; **6**: 49–60.
21. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidine-rich extract from pine (*Pinus maritima*) bark, Pycnogenol®. *Free Radic Biol Med* 1999; **27**: 704–724.
22. Fitzpatrick DF, Bing B, Rohdewald P. Endothelium-dependent vascular effects of Pycnogenol®. *J Cardiovasc Pharmacol* 1998; **32**: 509–515.
23. Rohdewald P. In: Coates PM, Blackman MR, Cragg G, Levine M, Moss J, White J. (eds) *Encyclopedia of Dietary Supplements*. New York: Marcel Dekker, 2005; 545–553.
24. Berryman AM, Maritim AC, Sanders RA, Watkins 3rd JB. Influence of treatment of diabetic rats with combinations of Pycnogenol, beta-carotene and alpha-lipoic acid on parameters of oxidative stress. *J Biochem Mol Toxicol* 2004; **18**: 345–352.
25. Kobayashi MS, Han D, Packer L. Antioxidants and herbal extracts protect HT-4 neuronal cells against glutamate-induced cytotoxicity. *Free Radic Res* 2000; **32**: 115–124.
26. Sastre J, Pallardo FV, Vina J. Glutathione, oxidative stress and aging. *Age* 1996; **19**: 129–139.
27. Dabrosin Ch, Öllinger K. Variability of glutathione during menstrual cycle – due to estrogen effects on hepatocytes? *Free Radic Biol Med* 2004; **36**: 145–151.
28. Kidd PM. Glutathione: systemic protectant against oxidative and free radical damage. *Altern Med Rev* 1997; **1**: 155-176.
29. Filomeni G, Rotilio G, Ciriolo MR. Cell signaling and the glutathione redox system. *Biochem Pharmacol* 2002; **64**: 1057–1064.
30. Asensi M, Garcia-España, Pallardó FV, Viña J, Estrela JM. Effect of non-protein thiols on protein synthesis in isolated rat hepatocytes. *Experientia* 1996; **52**: 111–114.
31. Schmitt-Schillig S, Schaffer S, Weber CC, Eckert GP, Müller WE Flavonoids and the aging brain. *J Physiol Pharmacol* 2005; **56**: 23–26.
32. Ferrer JV, Sastre J, Pallardó FV *et al*. Senile cataract: a review on free radical related pathogenesis and antioxidant prevention. *Arch Gerontol Geriatr* 1991; **13**: 51–59.
33. Sastre J, Asensi M, Gascó E *et al*. Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. *Am J Physiol* 1992; 263: 992–995.
34. Ďuračková Z, Križková L, Maruniaková A. Biological activities of natural polyphenols (Pycnogenol® and Egb761) and their interaction with vitamin C and E. XXI International Conference on Polyphenols. Morocco: Marrakech, 2002; 315–316.
35. Ďuračková Z, Rohdewald P, Muchová J *et al*. Supplementation with Pycnogenol® French maritime pine bark extract reduces markers of oxidative stress in children with attention deficit hyperactivity disorders (ADHD). 2nd International Conference on Polyphenols and Health. Supplemental Abstracts. Davis: University of California, 2005.
36. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. *Anal Biochem* 1980; **106**: 55–62.
37. Barnett V, Lewis, T. *Outliers in Statistical Data*, 3rd edn. New York: Wiley, 1994.
38. Lang CA, Mills BJ, Mastropaolo W, Liu MC. Blood glutathione decrease in chronic diseases. *J Lab Clin Med* 2001; **135**: 402–405.
39. Jacobsen JP, Rodriguiz RM, Mork A, Wetsel WC. Monoaminergic dysregulation in glutathione-deficient mice: possible relevance to schizophrenia? *Neuroscience* 2005; **132**: 1055–1072.
40. Castagne V, Rougemont M, Cuenod M, Do KQ. Low brain glutathione and ascorbic acid associated with dopamine uptake inhibition during rat's development induce long-term cognitive deficit: relevance to schizophrenia. *Neurobiol Dis* 2004; **15**:93–105.
41. Myhrstadt MC, Carlsen H, Nordstrom O, Blomhoff R, Moskaug JO. Flavonoids increase the intracellular glutathione level by transactivation of the γ-glutamylcysteine synthetase catalytical subunit promoter. *Free Radic Biol Med* 2002; **32**: 386–393.
42. Carlsen H, Myhrstad MC, Thoresen M, Moskaug JO, Blomhoff R. Berry intake increases the activity of the γ-glutamylcysteine synthetase promoter in transgenic reporter mice. *J Nutr* 2003; **133**: 2137–2140.
43. Ďuračková Z, Muchová J, Sivoňová M *et al*. Oxidative stress in pathophysiology of attention deficit hyperactivity disorder and its influence by a polyphenolic natural extract, Pycnogenol®. XXII International Conference on Polyphenols, Helsinki, Finland, August 25–28 2004, P37, 177–178.
44. Siler-Marsiglio KI, Paiva M, Madorsky I, Serrano Y, Neelez A, Heaton MB. Protective mechanisms of Pycnogenol® in ethanol-insulted cerebellar granule cells. *J Neurobiol* 2004; **61**: 267–276.
45. Wei YH, Peng QL, Lau BHS. Pycnogenol® enhances endothelial cell antioxidant defenses. *Redox Report* 1997; **3**: 219–224.