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Effect of polyphenolic extract, Pycnogenol®, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder

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Abstract

The purpose of this randomized, double-blind and placebo controlled study was to test the effect of polyphenolic extract of pine bark Pycnogenol® (Pyc) on the level of oxidized purines represented by 8-oxo-7,8-dihydroguanine (8-oxoG) and on the total antioxidant status (TAS) in children with attention deficit/hyperactivity disorder (ADHD).

We have found significantly increased damage to DNA in ADHD children when compared to controls. 8-oxoG was significantly lower after 1 month of Pyc administration in comparison to the beginning state and to placebo group. TAS in ADHD children was lower in comparison to controls. After Pyc administration, TAS was elevated but statistically significant increase was recorded after 1 month of termination of Pyc application. Improvement of DNA damage and TAS after Pyc administration is associated with the improvement of attention in ADHD children.

In conclusion, Pycnogenol® administration reduces oxidative damage to DNA, normalizes TAS and improves attention of ADHD children. Explanation of mutual relation between oxidative damage to DNA, TAS and symptoms of ADHD and mechanism of Pyc's action needs further investigations.

Keywords: *ADHD, Pycnogenol®, 8-oxoG, oxidized purines, TAS*

Abbreviations: *ADHD, attention deficit/hyperactivity disorder; 8-oxoG, 8-oxo-7,8-dihydroguanine; TAS, total antioxidant status*

Introduction

Oxygen derived free radicals are produced continuously inside the living cell. If they get out of control, they can cause a widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, DNA base modifications and polynucleotide strand breaks [1,2]. Hydroxyl radical may oxidatively modify the guanine or deoxyguanosine of nuclear and mitochondrial DNA to 8-oxo-7,8-dihydroguanine (8-oxoG) or 8-oxodeoxyguanosine (8-oxodG), which are the most commonly measured

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markers of oxidative DNA damage [1]. For evaluation of 8-oxoG, formamidopyrimidine-DNA glycosylase (Fpg) is commonly used [3], which converts oxidized purines to strand breaks determined by comet assay.

Excessive production of reactive oxygen species, at the deficiency of antioxidant defence, may be involved in pathogenesis of several diseases such as neurodegenerative diseases [4].

Attention deficit/hyperactivity disorder (ADHD) belongs to the most common neurodevelopmental disorders in children. It is characterized by the symptoms of distractibility, impulsivity and hyperactivity. The molecular basis of ADHD pathogenesis is not clear but it is predicted that genetic as well as nongenetic factors play an important role. It is assumed that amongst other factors, oxidative stress can contribute to the pathogenesis of ADHD [5–7]. Children with ADHD had an imbalance in catecholamine metabolism which can modify attention, the way of thinking and reactivity of receptors for catecholamines [8]. This disequillibrium may contribute to the oxidative stress in patients with ADHD [9].

Pycnogenol® (Pyc) is a standardized bark extract of the French maritime pine (*Pinus pinaster*). It consists mainly of procyanidins, catechin and phenolic acids [10]. Pyc has strong free radical—scavenging activity against reactive oxygen and nitrogen species and a wide range of positive effects *in vitro* and *in vivo* [11]. First case reports about positive effects of Pyc supplementation to children with ADHD were collected by Passwater [12] or by other authors [5,13,14]. In our study, we have found also a significant improvement of ADHD symptoms, inattention and hyperactivity, in children after 1 month of Pyc administration [15].

We assume that children suffering from ADHD can be under the oxidative stress and antioxidants such as Pyc can play a positive role in the treatment of the disease.

The aim of our study was to investigate whether damage to DNA plays a role in pathophysiology of ADHD and to examine the effect of Pyc on the level of 8-oxoG, a marker of oxidative damage to DNA and on the total antioxidant status (TAS) in children with ADHD.

Materials and methods

Patients

Sixty-one outpatients—50 boys and 11 girls—with ADHD, treated at the Department of Child Psychiatry of the Child University Hospital, Bratislava, Slovakia, age 6–14 years (average age 11.5) were enrolled in a randomized, double-blind and placebo controlled study [15].

Patients with acute inflammatory diseases, renal and cardiovascular disorders and diabetics were excluded from this study.

Pyc (1 mg/kg of body weight/day) or placebo were administered to children for 1 month. Placebo contained lactose (58 mg) and cellulose (65 mg) in the tablet. The same manufacturer, Drug Research Institute, Modra, Slovakia, produced tablets of Pyc and placebo.

Selection into the group (Pyc or placebo) was carefully randomized as described by Trebaticka et al. [15].

Patients were investigated at the beginning of 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).

Patients had a standard diet. They were not supplemented with any other psychotropic drugs or with other antioxidants such as vitamins E and C during the study.

The control group was represented by healthy children volunteers of the similar age (average age 11.5).

The Ethical Committee of the Child University Hospital approved the study. Parents gave a written consent for participation of their children in the study.

Sample collection

Venous blood samples were taken at start, after treatment and after wash-out period into commercial tubes with sodium citrate as an anticoagulant. Blood was used for immediate analyses by comet assay. Then blood samples were centrifuged and plasma was aliquoted, shock frozen and stored at − 80°C until further analysis.

Detection of DNA damage (comet assay)

Damage to DNA was determined in healthy controls and compared with ADHD children by single cell gel electrophoresis (comet assay) without enzymatic modification according to Collins et al. [16]. DNA damage was expressed as total damage (TD) that was calculated for each gel by using the following formula in which i is a class of damage (see farther) and N is the number of cells in each class:

$$\mathrm{TD} = \sum_{i=0}^{4} iN_i$$

For determination of oxidative damage to DNA in ADHD patients, enzymatically modified comet assay using formamidopyrimidine-DNA glycosylase (Fpg) which cleaves oxidatively modified purines [17–19], was used. Fpg has 8-oxoG as its main substrate, but it also detects formamidopyrimidines FapyA and FapyG, which are formed in much lower amount than 8-oxoG [20]. However, we used the same method to compare the oxidative damage to DNA before and after administration of the substance, thus possible positive

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results will be reflected in the same degree at both stages.

Briefly, lymphocytes were isolated from blood by standard centrifugation over a cushion of Histopaque and washed once with phosphate buffer saline, pH 7.4. The pellet of cells was suspended in 1% low melting point agarose in PBS and quickly placed on a glass microscope slide precoated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4°C and then the slides were immersed in lysis solution (2.56 M NaCl, 0.1 M EDTA, 10 mM Tris at pH 10, 1% Triton X-100) at 4°C for 1 h in order to remove cellular proteins and lipids. After washing in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0), the gels were incubated with formamidopyrimidine-DNA glycosylase (Fpg) protein in enzyme buffer or in enzyme buffer at 37°C only. Gels were then treated with alkaline buffer (0.3 M NaOH, 1 mM EDTA) (alkaline unwinding) for 40 min before electrophoresis (30 min) at 25 V, 300 mA in alkaline buffer at 4°C. The slides were then neutralized by washing three times for 5 min each with 0.4 M Tris/HCl, pH 7.5 at 4°C before staining with DAPI (4,6-diaminidino-2-phenylindole).

Image analysis and scoring

Images from simple as well as from Fpg-modified comet assay were scored using a fluorescence microscope (Olympus BX 41 with a 515–560 nm excitation filter and a 530 nm barrier filter). The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected comets. The comet-like DNA formations were categorized into five classes (0, 1, 2, 3 and 4), according to the intensity of DNA fluorescence in the tail relative to the head and total damage (TD) for each gel was calculated by the same formula as for simple comet assay.

The levels of 8-oxoG were calculated from values of TD using calibration curve $y = 134.97x + 7.0612$ according to ESCODD [21], where y means TD and x means breaks of DNA. From breaks, 8-oxoG per 10^6 guanine were calculated according to ESCODD [21]. Experiments were done in duplicate.

Total antioxidant status (TAS)

TAS in plasma was analysed using a kit Randox (United Kingdom) on Hitachi 911 automatic analyser. The TAS concentration is expressed in mM of plasma using Trolox as a standard.

Determination of biochemical parameters

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

Statistical analysis

Means ± SEM are given for the normally distributed parameters or as a median and interquartile range for data showing departures from normality (according to Shapiro-Wilk's test). Data, which showed no departures from normality, were analysed with the Student's t test for independent or pair-matched samples. To test for dual effects (therapy and duration) we used two-way ANOVA with repeated measures. For the evaluation of the total damage to DNA between the healthy and ADHD groups Mann–Whitney test was used as a non-parametric rank-based method for the comparison of two independent random samples.

Spearman's rank correlations were used to determine associations between 8-oxoG and TAS. Moreover, all pairwise comparisons among them and clinical parameters and among 8-oxoG and uric acid, catecholamines and clinical parameters were made.

For statistical analysis, we employed statistical program StatsDirect® 2.3.7 (StatsDirect Sales, Sale, Cheshire M33 3UY, UK). Graphical representation of data was made using program Excel 2000 (Microsoft Co.).

Results

We are the first who found significantly increased levels of total damage to DNA determined by simple comet assay in patients with ADHD in comparison to healthy controls of similar age at the beginning of the trial (198.5 vs 61.7 total damage score, $p < 0.001$) (Figure 1).

We found statistically decreased levels of 8-oxoG, as a main representative of all oxidatively damaged purines, (0.412 8-oxoG/10^6G) 1 month after Pyc administration in comparison to values before Pyc administration (0.558 8-oxoG/10^6G, $p = 0.012$). In placebo group, we have not found any significant change in 8-oxoG levels. When we compared the level of 8-oxoG in Pyc and placebo group after Pyc administration (period 1) we found the significant difference (0.412 8-oxoG/10^6G for Pyc vs 0.638 8-oxoG/10^6G for placebo group, $p = 0.014$). After the wash-out period (1 month after termination of Pyc administration, period 2) the level of 8-oxoG rose again (Figure 2).

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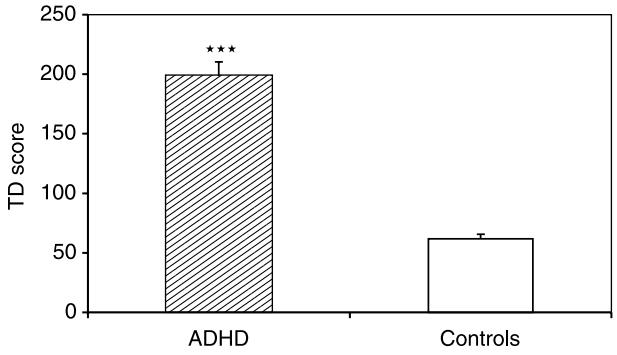

Figure 1. TD to DNA between the group of 58 children suffering from ADHD and 56 healthy children (controls) before Pyc/placebo administration (period 0), $p < 0.001$.

We found decreased TAS in plasma of children with ADHD (1.026 ± 0.021 mM) when compared to the reference values (1.1–1.7 mM). After 1 month of Pyc administration, TAS increased (1.050 ± 0.016 mM), however, this elevation was not statistically significant ($p = 0.119$). Statistically significant difference was found after the wash-out period (1.091 ± 0.021 mM in period 2 vs 1.026 ± 0.021 mM in period 0, $p = 0.0019$) (Figure 3). Placebo administration had no effect on TAS.

Basic biochemical parameters (bilirubin, glucose, gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, uric acid and lipid profile) were analysed in plasma. All values were in the physiological range before the trial, 1 month after Pyc or placebo administration and after the wash-out period, as reported by Trebaticka et al. [15].

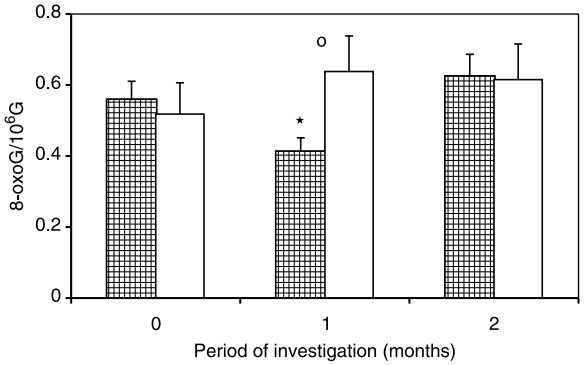

Figure 2. The level of 8-oxoG/10^6G in lymphocytes of children suffering from ADHD in Pyc group (grid column) ($n = 39$) or placebo group (empty column) ($n = 17$) during different periods of investigation. Values represent mean ± SEM. Period 0—examination before the trial, 1—one month after Pyc/placebo administration and 2—one month after termination of Pyc/placebo administration (wash-out period). ⋆—significance between examination 0 and 1 in Pyc group ($p < 0.05$); ○—significance between Pyc and placebo group in period 1 ($p < 0.05$).

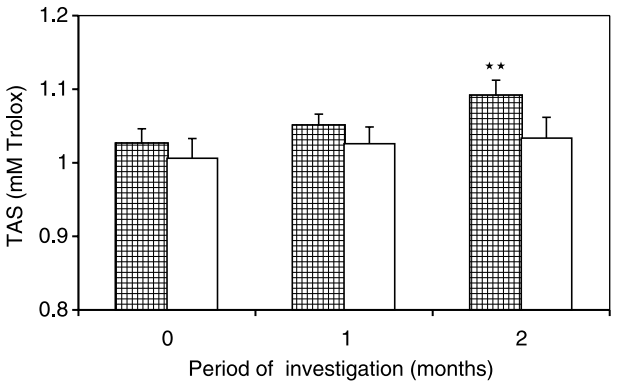

Figure 3. TAS expressed in equivalents of Trolox antioxidant ability (mM) in children suffering from ADHD after Pyc (grid column) ($n = 41$) or placebo (empty column) ($n = 17$) administration. Values represent mean ± SEM. Period 0—examination before the trial, 1—one month after Pyc/placebo administration and 2—one month after termination of Pyc/placebo administration. ⋆⋆—significance between period 0 and 2 in Pyc group ($p < 0.01$).

The levels of uric acid in Pycnogenol and placebo groups are given in Table I. No significant change in any investigated period in both groups were found.

We calculated associations between individual parameters, 8-oxoG and TAS as well as between them and clinical parameters. We found no associations between 8-oxoG level and clinical parameters before the trial. However, after Pyc administration we found associations between 8-oxoG, TAS and inattention in ADHD children. Correlation between 8-oxoG differences in period 1 and 0 and inattention characterised by the score according to Trebaticka et al. [15] is depicted in Figure 4A. In Figure 4B there is a correlation between TAS differences and inattention score [15]. In placebo group, no correlation was found.

We found a weak negative association between TAS and 8-oxoG ($y = -0.082x + 1.069$, $r = -0.217$, $n = 54$, $p = 0.048$) before the trial. The significance was lost after Pyc administration.

Discussion

One of alternative treatments of children with ADHD involves administration of extract of French maritime pine bark—Pycnogenol®. One-month of Pyc administration caused a significant reduction of hyperactivity, improved attention and visual-motoric coordination and concentration of children with ADHD. In the placebo group, no positive effects were found. One month after termination of Pyc administration, a relapse of symptoms was noted [15]. The molecular basis of positive effects of Pyc on ADHD symptoms is unknown. Pyc is a complex mixture of phenolic substances and exhibits a wide variety of interesting biochemical and pharmaco-

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Table I. Uric acid levels (μM) in children suffering from ADHD after Pyc ($n = 41$) and placebo ($n = 16$) administration.

	Uric acid (μM)		
Months of investigation	0	1	2
Pycnogenol group	205.76 ± 5.61	200.96 ± 7.01	209.58 ± 7.25
Placebo group	216.49 ± 11.67	210.22 ± 11.49	234.06 ± 15.77

Values represent mean ± SEM. Period 0—examination before the trial; 1—one month after Pyc/placebo administration and 2—one month after termination of Pyc/placebo administration.

logical properties [11]. The main components of Pyc extract are procyanidine oligomers with chain length between 2 and 12 monomeric units. They may interact with DNA molecule attacked by free radicals and reactive oxygen and nitrogen species, which can play an important role in the development and progression of neurodevelopmental disorders [4].

It is known that children with ADHD have disturbance in catecholamine metabolism. In our pilot study, we also detected increased levels of catecholamines (adrenaline, noradrenaline and dopamine) in urine of ADHD children in comparison to healthy children [22]. We also found a positive association between dopamine and noradrenaline levels and clinical symptoms characterised by hyperactivity evaluated by teacher rating scale (CTRS) questionnaire [15] ($r = 0.361$, $p = 0.041$ and $r = 0.318$, $p = 0.031$, respectively). After the Pyc administration, both associations were abolished (Dvorakova et al., unpublished results), which supports our assumption of participation of malfunction in catecholamine metabolism in pathogenesis of ADHD. During autooxidation of catecholamines superoxide anion and H_2O_2 can be formed. Both reactive oxygen species trigger hydroxyl radical formation, which can initiate oxidative damage to DNA [9].

Nelson et al. [23] have studied *in vitro* ability of Pyc to protect DNA from iron/ascorbate—induced damage and have reported a decrease in single and double strand DNA breaks. They suggested that Pyc can play potentially important role in protection of genetic material against free radical—induced injury. These results *in vitro* are confirmed by our results

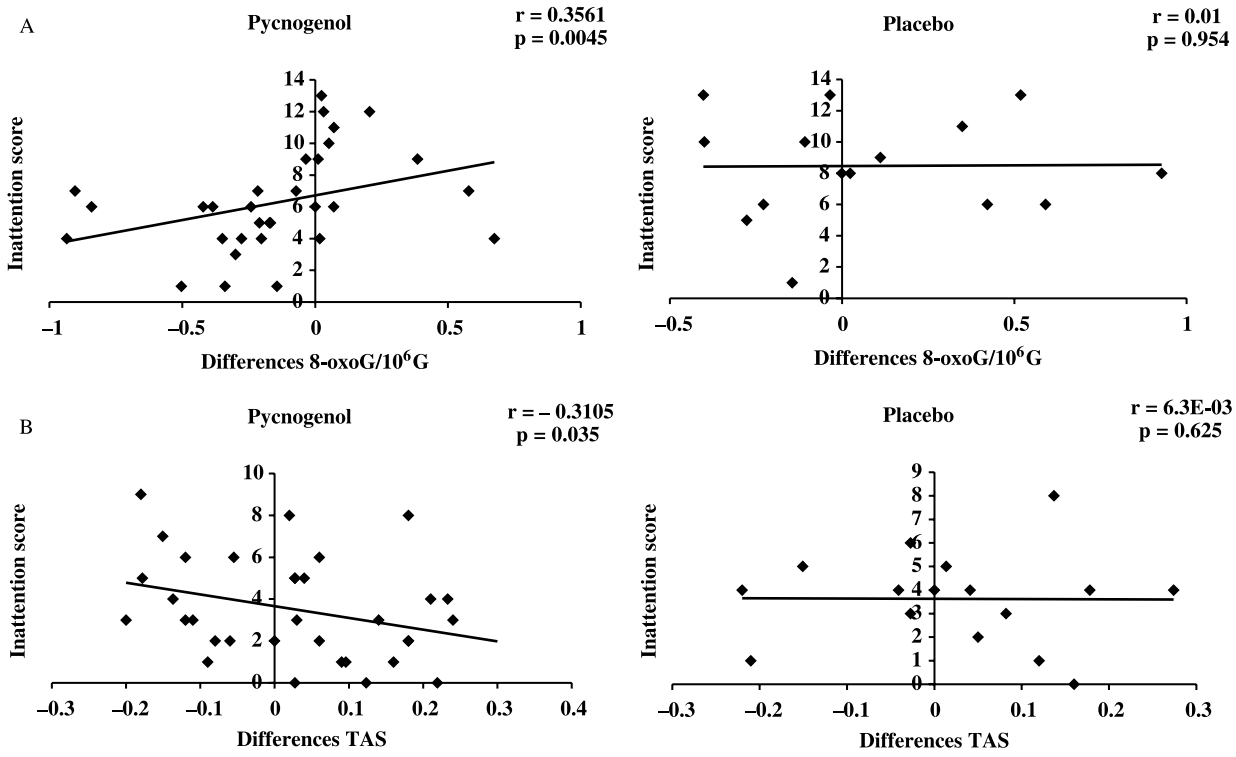

Figure 4. (A) Correlations between inattention score [27] in period 1 and 8-oxoG/10^6 G differences between periods 1 and 0 in Pycnogenol ($y = 3.105x + 6.718$, $n = 31$) and placebo ($y = 0.089x + 8.459$, $n = 15$) groups. Period 0—examination before the trial, period 1—one month after Pyc/placebo administration. (B) Correlations between inattention score [27] in period 1 and TAS differences between periods 1 and 0 in Pycnogenol ($y = -5.612x + 3.662$, $n = 33$) and placebo ($y = -0.092x + 3.627$, $n = 16$) groups. Period 0—examination before the trial, period 1—one month after Pyc/placebo administration.

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obtained *in vivo*. We found that children with ADHD had increased damage to DNA in comparison to healthy children. We found significantly decreased level of oxidatively modified purines after 1 month of Pyc administration to children suffering from ADHD from which 8-oxoG is the main oxidative product [20]. However, we found no correlations between 8-oxoG and clinical parameters before the trial.

We have found a positive association between inhibition of 8-oxoG formations determined as 8-oxoG differences between period 1 and 0 by administered Pyc and improvement of attention in ADHD children (Figure 4A). Placebo administration exhibits no association. Results characterising improvement of ADHD symptoms after Pyc administration were published recently by Trebaticka et al. [15].

We assume that modification of DNA found in ADHD children is not primarily linked with pathology of ADHD. As we found a weak but significant association between TAS and 8-oxoG, increased damage to DNA could be the result of oxidative stress. We do not know the exact mechanism of Pyc's protective effect against DNA damage. We suppose that a direct scavenging activity, chelating ability, stimulation of DNA repair system or their combinations may be involved. Taubert et al. [24] found that a direct antioxidant activity of flavonoids *in vivo* is unlikely because the rate constants of free radical reactions with the target molecules are much higher than those with flavonoids. We have not confirmed an increased antioxidant ability of plasma in children with ADHD after 1 month of Pyc administration. We suppose that only direct antioxidant activity of Pyc is not responsible for the inhibition of 8-oxoG formations after Pyc administration. Collins et al. [25] reported that flavonoids present in kiwi fruit could increase the repair ability of DNA of volunteers *in vivo* and *ex vivo*. Whether Pyc possesses similar ability to stimulate DNA repair ability as polyphenols in kiwi still remains a question.

Another unknown is determination of relation between inhibition of 8-oxoG level after Pyc administration and improvement of attention of ADHD children. Significantly higher level of DNA damage in leukocytes of mild cognitive impairment (MCI) patients have found Migliore et al. [26] and significant elevation of 8-hydroxyguanine in brain of post mortem MCI patients was detected by Wang et al. [27]. Whether it is a causal relationship between the level of the marker of oxidative damage to DNA and attention or it is a consequence of inhibition of oxidative stress, it still remains a question. From our results it follows that TAS in children with ADHD is slightly decreased when compared with reference values of the healthy individuals. Pyc administration caused a slight but not significant elevation of the TAS. In the contrary to placebo administration, after Pyc administration we found a correlation between increased TAS and improved attention, similarly as we have seen in case of 8-oxoG. However, the exact mechanism of these relationships is not clear yet.

It was interesting to find out, that improvement of the antioxidant status persisted also after the wash-out period when the increase was significant in comparison to period 0. In spite of the fact that Pyc exerts a marked antioxidant activity *in vitro* in hydrophilic as well as in lipophilic environment [28], 1 month of Pyc administration to children with ADHD in doses 1 mg/kg/day caused only the mild, nonsignificant increase in antioxidant capacity *in vivo*. It is obvious when we realize, that the mean concentration of polyphenols in blood is around 10 μM, which is calculated from administered Pyc in doses 1 mg/kg of body weight, from average molecule weight of individual polyphenols present in Pyc (in average 300), and from around 30% absorption of polyphenols in gastrointestinal tract. Children have higher concentration of other antioxidants in blood (e.g. ascorbic acid—around 50 μM, tocopherols 30 μM and uric acid 100–260 μM) than calculated Pyc contribution. From this follows, that the direct Pyc contribution to antioxidant capacity of plasma is negligible when compared with other plasma antioxidants.

We investigated also the level of uric acid, although it is assumed that in children uric acid contributes to the total antioxidant ability by lower share than in adults. As uric acid levels have not changed after 1 month of Pyc/Placebo administration (Table I) we assume that uric acid is not responsible for the inhibition of 8-oxoG formations. The same result is also obvious from nonsignificant correlations between uric acid and 8-oxoG levels at all examined periods. However, we found a weak, but significant negative correlation between TAS and 8-oxoG levels ($r = -0.218$, $n = 54$, $p = 0.048$). Due to the fact that 8-oxoG level is a result of the balance between the action of oxidants on DNA, antioxidative protection and activity of DNA repair systems [29], it is not clear which component of that complicated system participates in it primarily.

In addition to the direct antioxidant activity, Pyc has the ability to stimulate production of antioxidant enzyme such as SOD [30], through both upregulation of Cu/Zn SOD protein expression [31] and increasing its activity [32]. Stimulation of SOD expression needs some time and might persist even after discontinuation of polyphenols administration. Nelson et al. [33] found that polyphenolic extract of five medicinal plants needed up to 120 days of extract administration to increase erythrocyte SOD activity by 30%. Even though SOD is not a main plasma antioxidant, its elevated activity in cells may save low-molecular weight antioxidants, leading to the increase of their activity in plasma, which persists also after termination of Pyc administration [29]. Pyc also regenerates

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other antioxidant systems indirectly, for example, through the increase of glutathione reductase activity [34]. In this way, regenerated glutathione (GSH) can also contribute to TAS and cause its increase. We have also found that after wash-out period (period 2) the level of reduced glutathione significantly increased (Dvorakova et al., unpublished results) in comparison to its level at the beginning of the trial. Regenerated glutathione can also contribute to the increased TAS in period 2.

In conclusion, Pycnogenol® administration reduces oxidative damage to DNA in children with ADHD and normalizes their TAS, which both are related to the improvement of attention of ADHD children. However, explanation of mutual relation between oxidative damage to DNA, TAS and symptoms of ADHD such as inattention, as well as, a precise mechanism of Pyc's action will need further investigations.

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