

# How Does Pycnogenol<sup>®</sup> Influence Oxidative Damage to DNA and Its Repair Ability in Elderly People?

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Received September 3, 2010; Accepted November 11, 2010.

**Key words:** Oxidative damage and repair ability of DNA – Pycnogenol – Elderly people

**Abstract:** Our purpose in this randomized, double blind, placebo controlled study was to find out the possible effect of a polyphenolic pine bark extract, Pycnogenol<sup>®</sup> (Pyc) on the level of 8-oxo-7,8-dihydroguanine (8-oxoG) as representative of oxidative damage to DNA and on the DNA repair ability of elderly people. According to our results, three months of Pyc administration had no effect on the level of oxidative damage to DNA or on repair ability, but we found a relationship between the level of 8-oxoG and repair ability of DNA in this group. To conclude, even if the positive effect of Pyc was not confirmed in the case of elderly people it is important to highlight the necessity of further investigations about the mechanisms of Pyc acting on different age groups.

*This study was supported by VEGA grants 1/2294/05 and 1/0224/08, Socrates/Erasmus project (2006/2007) and UK travel grant (3/2006).*

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## Introduction

It is generally considered that oxidative stress, defined as an imbalance in free radicals production and their elimination, is involved in the damage to biological macromolecules, such as proteins, lipids and nucleic acids (Halliwell and Gutteridge, 1999; Collins and Dušinská, 2002). These changes are considered to be a cause of many disorders, including diabetes mellitus (Országhová et al., 2009), erectile dysfunction (Galiano et al., 2010), and attention deficit hyperactivity disorder (ADHD) (Chovanová et al., 2006). In particular, damage to nuclear or mitochondrial DNA can lead to mutations and subsequent carcinogenesis.

The most commonly measured marker of oxidative damage to DNA is 8-oxo-7,8-dihydroguanine (8-oxoG) (Halliwell and Gutteridge, 1999). Formamidopyrimidine DNA glycosylase (FPG) is an enzyme which recognizes 8-oxoG (Collins et al., 2004). By its action, the oxidized base is removed and converted to a break, which can be measured by the comet assay.

It is thought that oxidative damage to DNA might rise with increasing age. According to the DNA damage theory of aging, aging is a consequence of unrepaired DNA damage accumulation. In normal living cells, most of the damage is removed before replication of the DNA can convert it to mutations. 8-oxoG and other oxidized bases are repaired mostly by the base excision repair pathway (BER) (Collins, 2009). BER differs from nucleotide excision repair (NER), which deals with bulky adducts and leads to removal of an oligonucleotide of ~28 bases. In contrast to BER, defects in NER in humans can lead to rare genetic diseases such as xeroderma pigmentosum (Fan and Luo, 2010).

In general, we could predict that individuals with a high repair capacity should show lower DNA damage; but on the other hand, there is a second possibility, that a high level of damage induces a higher repair enzyme activity (Collins, 2009). Further investigations of this problem are needed.

Oxidative damage to DNA and DNA repair ability might be influenced by natural polyphenols (Chovanová et al., 2006; Zheng et al., 2010). These compounds, found in green and black teas, fruits and vegetables, olive oil, red wines and chocolate have been shown to be excellent antioxidants. In addition to their antioxidant functions, they exert anti-inflammatory, vasodilatory, and cardiovascular effects, and they are also implicated in the prevention of neurodegeneration and prevention of cancer (Perron et al., 2008).

Pycnogenol<sup>®</sup> (Pyc) is a standardized pine bark extract from French maritime pine (*Pinus pinaster*). It represents a blend of catechin, epicatechin, taxifolin, procyanidins and phenolic acids. Pyc has strong free radical-scavenging activity *in vitro* and it stimulates the synthesis of antioxidant enzymes (Rohdewald, 2005).

A positive effect of Pyc on DNA damage in ADHD children was already described by Chovanová et al. (2006). The aim of our study was to investigate whether the effect of Pyc on the level of 8-oxoG as a marker of oxidative damage

to DNA in elderly people is comparable to the effect in children and whether the repair ability of DNA can be influenced by Pyc.

## **Material and Methods**

### *Patients*

Fifty four patients – 41 women and 13 men with diagnosed osteoarthritis (OA) of knees, treated at the 2<sup>nd</sup> Department of Orthopaedics of the Comenius University Faculty of Medicine, University Hospital Ružinov, with average age  $54.0 \pm 1.0$  year, were enrolled in a randomized, double-blind and placebo (PL) controlled study.

The Ethical Committee of the University Hospital in Bratislava approved the study.

### *Medication*

Patients were supplemented with either Pyc at the dose of 150 mg/day or PL with identical shape and appearance and same number of pills/day as in the case of Pyc for three months. PL contained lactose (58 mg) and cellulose (65 mg) in tablet. Both, Pyc and PL tablets were produced by Manhattan Drug Co., NY, USA.

Selection into the Pyc or PL group was carefully randomized. The ratio of these groups was 1:1. The sample size was estimated assuming the power of 80% (beta of 20%), the type one error (alpha) of 5%. We included to the study 28 patients to Pyc group and 26 patients to PL group. Stat Direct 2.3.7 was used for the randomization an unpaired random allocation to intervention or control group and for the sample size estimation.

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

### *Methods*

Patients were investigated at the beginning of the trial before Pyc/PL administration, after three months of Pyc/PL administration and one month after termination of treatments (wash-out period).

Venous blood samples were taken into commercial tubes with sodium citrate as anticoagulant. Lymphocytes were isolated from blood by centrifugation on a density gradient and used for determination of oxidative damage to DNA and DNA repair ability. Simultaneously, aliquots of blood samples were centrifuged; plasma was shock-frozen in liquid nitrogen and stored at  $-80$  °C until further analysis.

### *Detection of DNA damage (comet assay)*

Damage to DNA in lymphocytes was determined by single cell gel electrophoresis (comet assay) according to Collins et al. (1997).

The levels of 8-oxoG were calculated from Fpg sites, represented by total damage (TD) values reduced about buffer score, where in TD  $i$  is a class of damage and  $N$  is the number of cells in each class:

$$TD = \sum_{i=0}^4 i \times N_i$$

by using the calibration curve  $y = 134.97x + 7.0612$  (ESCODD, 2004), where  $y$  means number of Fpg sites and  $x$  means breaks of DNA. The concentration of 8-oxoG per  $10^6$  guanines was calculated as described (ESCODD, 2004). Experiments were done in duplicate.

#### Repair assay

The modified comet assay was used to measure *in vitro* repair (Collins et al., 2001).

HeLa cells treated with Ro 19-8022 and light to induce 8-oxoG, embedded in agarose, provide the damaged DNA substrate for incubation with extracts from isolated lymphocytes prepared as described (Collins et al., 2001).

Based on the intensity of DNA fluorescence, 100 comets/gel were classified into five classes and the total score was calculated. This score is proportional to DNA break frequency. Total damage was expressed as % of damage caused by Fpg enzyme (control slide).

#### Statistical analysis

Descriptive statistics were obtained for all variables using mean  $\pm$  SEM for normally distributed continuous variables. Categorical variables were described using frequencies and proportions. 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 associations between comet assay and repair ability were analyzed with Spearman's correlations.

For statistical analysis, we employed the statistical program StatsDirectR 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,  $\gamma$ -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 Pycnogenol<sup>®</sup> or placebo administration.

At the beginning of the study, the level of 8-oxoG/ $10^6$ G for the Pycnogenol<sup>®</sup> group was  $1.2 \pm 0.1$ . One month of Pyc administration caused no significant change in 8-oxoG ( $1.3 \pm 0.1$  8-oxoG/ $10^6$ G;  $P > 0.05$ ). Similarly, in the placebo group, no significant change was observed ( $1.081 \pm 0.166$  vs.  $1.4 \pm 0.2$  8-oxoG/ $10^6$ G;  $P > 0.05$ ) three months after placebo administration. After the wash-out period, the level of 8-oxoG/ $10^6$ G remained the same in both Pyc ( $1.2 \pm 0.144$  8-oxoG/ $10^6$ G;  $P > 0.05$ ) and placebo group ( $1.3 \pm 0.134$  8-oxoG/ $10^6$ G;  $P > 0.05$ ) (Figure 1).

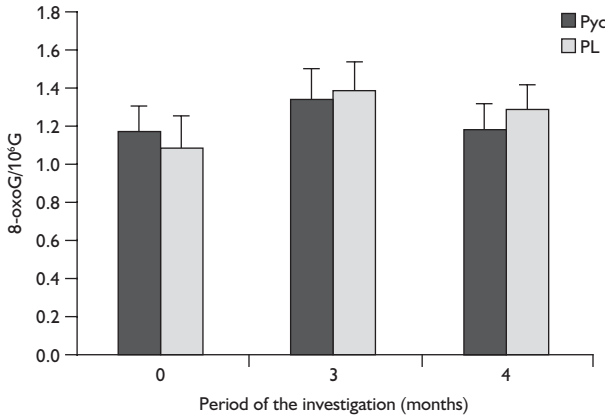


Figure 1 – Levels of 8-oxoG/10<sup>6</sup>G in lymphocytes of elderly people in Pyc group (dark column) (n=28) or placebo group (light column) (n=26) during different periods of the investigation. Values represent mean ± SEM. Period 0 – examination before the trial, 3 – three months after Pyc/placebo administration and 4 – one months after termination of Pyc/placebo administration (wash-out period).

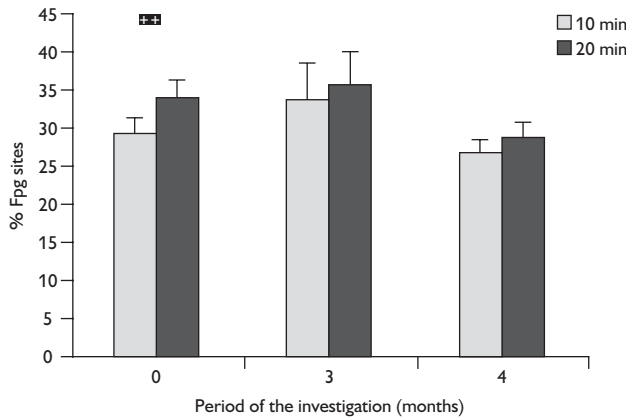
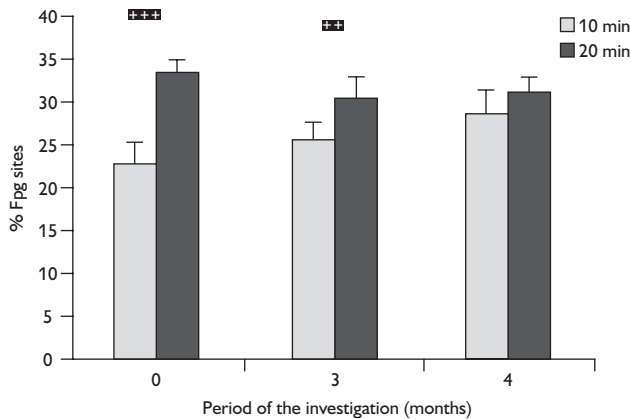


Figure 2a and b – Repair ability of DNA in lymphocytes of elderly people in Pyc (a) (n=24) and placebo group (b) (n=24) after 10 min (light column) and 20 min (dark column) incubation of reparative enzymes with damaged DNA of HeLa cells during different periods of the investigation. Values represent mean ± SEM. Period 0 – examination before the trial, 3 – three months after Pyc/placebo administration and 4 – one months after termination of Pyc/placebo administration (wash-out period). \*\*\* – statistical significance in period 0 or 3 between 10 or 20 min of incubation (P<0.01) \*\*\*\* – statistical significance in period 0 between 10 or 20 min of incubation (P<0.001)



To determine the DNA repair ability of lymphocyte extract, a modified comet assay was used. Repair ability was measured by incubating HeLa cell substrate containing oxidatively damaged bases DNA with lymphocyte extracts. Repair ability was measured at different times of incubation (10 and 20 min), as cleavage of damaged bases by endonucleases and increase of AP sites. Repair ability of our patients was expressed as % of activity of Fpg on the same substrate.

In case of 10 min of incubation, before Pyc/PL administration, TD in Pyc was  $29.23 \pm 2.12\%$ , in placebo group  $24.2 \pm 2.179\%$ . Three month of Pyc administration had no effect on TD ( $33.66 \pm 4.638\%$ ;  $P > 0.05$ ). In placebo group, TD after 3 months of administration was  $25.8 \pm 1.834\%$ ;  $P > 0.05$  (Figure 2a and b).

In case of 20 min of incubation, before Pyc/PL administration, TD in Pyc was  $33.75 \pm 2.434\%$ , in placebo group  $33.4 \pm 1.572\%$ . After Pyc administration, TD was not changed ( $35.45 \pm 4.401\%$ ;  $P > 0.05$ ). Similar results were found in placebo group ( $30.5 \pm 2.38\%$ ;  $P > 0.05$ ) (Figure 2a and b).

We found a significant difference in 10 and 20 min incubation of extract with damaged DNA of substrate in both Pyc and placebo groups ( $P < 0.01$  in Pyc and  $P < 0.001$  in placebo group) (Figure 2a and b). Similarly, significant difference was found after three months of placebo intake ( $P = 0.01$ ) (Figure 2b).

Of interest is the positive correlation between 8-oxoG/10<sup>6</sup>G level and repair ability of DNA after 10 and 20 min of incubation of oxidative damaged DNA with extract from lymphocytes which among other things contained enzymes involved in DNA repair (Figure 3). The trend of positive correlation was found already after 10 min ( $P > 0.05$ ), and it becomes significant after 20 min of incubation with extract ( $P < 0.05$ ).

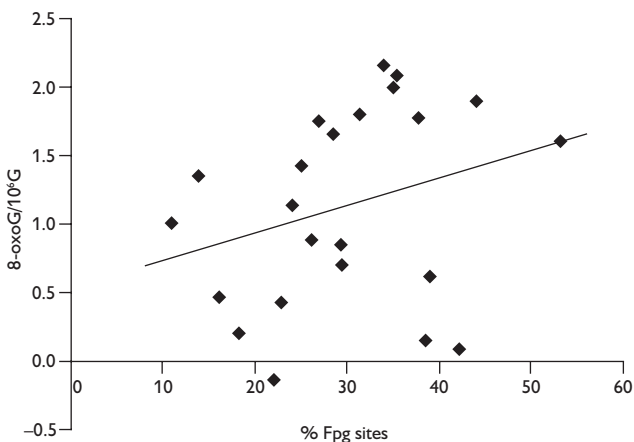


Figure 3 – Correlations analyzed before Pyc administration (period 0) between oxidative damage to DNA (8-oxoG/10<sup>6</sup>G) and repair ability to DNA (TD%) after 20 min incubation with extract ( $n = 22$ ,  $y = 0.0201x + 0.5305$ ,  $R = 0.289$ ,  $P = 0.0476$ ). Repair ability to DNA was expressed as %TD  $\pm$  SEM compared to Fpg enzyme, whose activity = 100%.

## Discussion

Population suffering from OA (group of people of higher age) was used to verify the effect of Pyc to the level of 8-oxoG/10<sup>6</sup>G and repair ability of DNA. We do not predict the relationship between osteoarthritis (OA) of knees and oxidative damage to DNA.

The comet assay represents a reliable method to determine the level of oxidative damage to DNA in the cell through strand break determination (Collins et al., 1997; Collins, 2004). The research teams in the European project ESCODD, studying DNA oxidation damage in lymphocytes, found that in the group of healthy individuals of 20–30 years of age the average level of 8-oxoG/10<sup>6</sup>G was 0.34 (ESCODD, 2004). At the beginning of our study, the damage to DNA was  $1.167 \pm 0.14$  8-oxoG/10<sup>6</sup>G. Since the average age of our group was 54.0 year and it is thought that in aging there is an accumulation of damage, it is not possible to compare these two results. For that reason it would be useful to find out the level of 8-oxoG of the healthy human of similar age. However, this needs further investigations.

In our study, no effect of Pyc to oxidative damage to DNA in elderly people was confirmed. An effect of Pyc intake in children with attention deficit hyperactivity disorder (ADHD) was observed by Chovanová et al. (2006). They found that after Pyc administration the level of 8-oxoG as the main marker of damaged purines significantly decreased in comparison to the level before Pyc administration. The higher average age of our group and the unknown molecular action of Pyc make it hard to predict the effect of polyphenols and the finding of the decrease of 8-oxoG in children is not in conflict with the present study.

Relatively low levels of DNA damage in healthy individuals (14) reflect the presence of antioxidants in the cells and the repair of DNA. Reactive oxygen species, which occur naturally within cells (e.g. in respiration), are eliminated by antioxidant enzymes. Damage and repair of DNA are in equilibrium and most damage is removed before the replication of the cell's DNA. Previous studies suggest that failures in DNA repair may lead to some rare disease formation, in which the failure in repair enzymes genes occurs (Fan and Luo, 2010).

To determine the repair ability of DNA of elderly people the modified comet assay was used (Collins et al., 2001). This method is based on measurement of the activity of repair enzymes in an extract prepared from lysed lymphocytes and incubated with a HeLa cells treated with photosensitizer and light and embedded in the agarose.

Activity of repair enzymes was examined as a function of the time of incubation with the substrate, oxidatively damaged DNA of HeLa cells. The intensity of repair was evaluated as the ability of a lymphocyte extract to excise damaged bases from DNA.

In comparison of 10 and 20 min of incubation of the extract with substrate DNA the trend of interindividual variability in 20 min was confirmed (Collins et al., 2001).

A possible explanation is that, at the beginning, until 10 min, the endonuclease activity predominates, whereas later, after 20 min of the incubation, other steps of DNA repair, including resynthesis of cleaved nucleotides, are involved.

Based on the fact that Pyc had no effect on DNA damage and on Bohr's (2002) findings that in ageing DNA repair ability declines, we did not predict the effect of Pyc on DNA repair ability. To confirm, three months of Pyc administration in our study did not influence the DNA reparation.

Interesting was finding of positive correlation between 8-oxoG/10<sup>6</sup>G and repair ability of DNA before Pyc administration. The uniform opinion about the relationship between DNA damage and its reparation still does not exist. It is still not known, if:

- a) low repair ability leads to increased oxidative damage to DNA (and on the contrary, the increased DNA repair to lower level of 8-oxoG/10<sup>6</sup>G),
- b) high oxidative damage induces the DNA repair, or
- c) there is no relationship between them and we can say, that damage to DNA and its repair proceed independently.

Our limited data suggest that proposition b) is most likely to apply.

Monitoring of oxidative damage to DNA and DNA repair ability and their possible modulation should lead to a better understanding of their involvement in the process of carcinogenesis, and possible routes to a decrease in incidence of cancer. Valid conclusions will be possible only after necessary further research.

*Acknowledgements: The authors thank to P. Cisár, MD., and R. Jáni, MD., from the 2<sup>nd</sup> Department of Orthopedics of the Comenius University for their work with patients and to L. Chandogová and L. Miková for their technical assistance.*

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