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Influence of oak wood polyphenols on cysteine, homocysteine and glutathione total levels and PON1 activities in human adult volunteers – a pilot study

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Abstract. Oxidative stress reflects an imbalance between antioxidants and pro-oxidants. Many diseases like atherosclerosis or heart failure are involved in oxidative stress. Increased oxidative stress is one of the potential contributing factors to aging. The aim of this study was to monitor the total thiol levels as markers of oxidative stress in 20 healthy volunteers after polyphenols intake (extract from the French oak wood *Quercus robur* – Robuvit® (300 mg/day)). Polyphenols are known as biomodulators with antioxidant activities. Homocysteine, cysteine and glutathione total levels were determined by using HPLC with electrochemical detection. The activity of the antioxidant enzyme paraoxonase-1 toward two substrates was determined by spectrophotometry. The level of thiol compounds and paraoxonase-1 activities were controlled after run-in (week 0), intervention (week 4) and washout (week 6) period. After the intervention period the results showed that Robuvit® had no significant influence on glutathione level ($p = 0.382$) and paraoxonase activities towards both, arylester and lactone substrates. On the other hand, homocysteine and cysteine levels decreased significantly ($p = 0.029$; $p < 0.001$, respectively). The negative correlation between paraoxonase lactonase activity and homocysteine level was noticed. This confirms that paraoxonase might play an important role in homocysteine-thiolactone metabolism.

Key words: Ellagitannins — Oxidative stress — Paraoxonase activity — Plasma aminothiols — *Quercus robur*

Abbreviations: ADMA, asymmetric dimethylarginine; Cys, cysteine; GSH, glutathione; HCy, homocysteine; LOD, limit of detection; LOQ, limit of quantification; PON1, paraoxonase-1; S/N, signal-to-noise.

Introduction

Plasma aminothiols, including homocysteine (HCy), cysteine (Cys) and glutathione (GSH) are investigated as potential indicators of redox state of the organism and disease risk. Increased plasma HCy is considered as an independent risk factor for cardiovascular disease including atherosclerosis and venous thrombosis. The participation of HCy in cardiovascular risk is not quite understood. HCy seems to cause endothelial dysfunction, induces LDL oxidation and thus leads to the formation of vascular foam cells, while being related with disturbances of blood coagulability (Marinou et al. 2005).

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HCy is a non-proteinogenic amino acid and was discovered in 1932. It was identified as a product of the essential amino acid methionine (Carmel and Jacobsen 2001). It can be remethylated to methionine or converted to Cys. Plasma HCy level is determined by multiple factors like genetic, demographic, acquired, and lifestyle determinants (Karolczak and Olas 2009). The usual concentration of total HCy reported in several studies of healthy humans varies between 5 and 15 μmol/l (Nekrassova et al. 2003).

Excess of HCy diffuses into the extracellular medium (Chwatko and Bald 2002). HCy is present in the organism in various forms. In the plasma, approximately 70% of HCy circulates bound to proteins, 25% is bonded with itself to form the dimer and less than 5% is bonded with other thiols, including Cys or is present in 1–2% in reduced form as thiol. The majority of HCy is present in oxidized form – mixed disulphide and homocysteine thiolactone (Isobe et al. 2005).

The thioester of homocysteine and HCy-thiolactone play an important role in atherothrombosis. It can form isopeptide bonds with protein lysine residues (homocysteinylation), which alters protein function, activates autoimmune response and enhances thrombosis. All mammals including human have mechanisms for elimination of thiolactone. One such mechanism is a calcium-dependent enzyme associated with HDL-lipoproteins, paraoxonase-1 (PON1), which hydrolyses HCy-thiolactone (Ďuračková and Andrezálová 2009; Jakubowski 2010). PON1 protects proteins against N-homocysteinylation, which is detrimental for protein structure and function and is related to endothelial dysfunction and atherogenesis (Macharia et al. 2012).

The increased level of Cys in physiological fluid such as plasma and urine has recently been recognized as an important indicator for several clinical disorders including cystinosis and cystinuria (Toyo´oka 2009). Cys is a non-essential proteinogenic amino acid involved in protein synthesis and in other metabolic processes of cells. Cys is one of the most abundant plasma thiol and its total plasma concentration is between 200–300 μmol/l (Rafii et al. 2009). It is a metabolic precursor of reduced glutathione (GSH) – an important "redox buffer" in the organism.

GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) and it is recognized as a key physiological antioxidant that not only detoxifies reactive species directly, but also enhances the functional ability of other crucial antioxidants through their regeneration (ascorbate) or as a co-factor of enzyme glutathione peroxidase. GSH is present in the organism in reduced (GSH) and oxidized (GSSG) form, where the level of GSH is 10–100 times higher than GSSG (Ďuračková 2014). GSH total level in plasma is about 6 μmol/l (Carmel and Jacobsen 2001; Ďuračková 2010).

The influence of polyphenols on aminothiol levels in human physiological fluids is unclear. The polyphenols in red wine (Chiva-Blanch et al. 2013), in cocoa (Andújar et al. 2012) or in black tea and coffee (Hodgson et al. 2003) did not show a significant influence on plasma HCy level. On the other hand, regular chokeberry juice drinking resulted in reduction of total HCy level (Skoczynska et al. 2007).

The aim of this study was to investigate whether intervention with polyphenolic extract from French oak wood consist namely of roburins, Robuvit® can modify levels of the cardiovascular risk factor, HCy. The other plasma thiols (Cys, GSH) and PON1 activities were also monitored. Roburins are very potent antioxidants (Bazylko et al. 2013; Fracassetti et al. 2013). Humans have been exposed to these polyphenols for centuries from wine and spirits that matured in oak wood barrels. Oak wood is currently the only known source of roburins and according to this specificity, the major source of roburins in human diets results from the consumption of wine and spirits (cognac and whiskey) traditionally matured, aged and stored in oak barrels.

Material and Methods

Material

Sodium phosphate monobasic monohydrate, sodium borohydride, perchloric acid, 1-octanesulfonic acid sodium salt, acetonitrile, L-cysteine, L-methionine, L-glutathione reduced and DL-homocysteine were obtained from Sigma Aldrich (Steinheim, Germany), ortho-phosphoric acid, DL-penicillamine from Fluka (Buchs, Switzerland), phenyl acetate and dihydrocoumarin were purchased from Sigma Aldrich (Missouri, USA).

Characteristic of Robuvit®

The French oak wood extract Robuvit® (Horphag Research Ltd.) is a registered proprietary water extract obtained from the wood of *Quercus robur*. The plant belongs to the plant family *Fagacae*, genus *Quercus*. The oak wood contains a specific profile of tannins named roburins that are part of the ellagitannins. Robuvit® is standardized and specified to contain at least 20% of roburins (A, B, C, D, E) including grandinin. The two most abundant ellagitannins in the Robuvit® are stereoisomers vescalagin and castalagin (Figure 1), which were originally isolated and described by Mayer et al. (1967). Roburins and grandinin are dimers of these compounds or differ by the presence of a pentose substituent. Further to the roburins, Robuvit® contains monomeric vescalagin and castalagin as well as ellagic acid and gallic acid. Robuvit® is bioavailable to humans and its consumption is associated with increase of antioxidant capacity at hydrophilic conditions (Natella et al. 2014).

Study design and sample

Twenty healthy non-smoking volunteers (8 men and 12 women, aged 44–65 year) were included in the study. Volunteers signed informed agreement before the study. The study was approved by the Ethical committee of the Medical Faculty of Comenius University and University hospital, Bratislava.

Volunteers were instructed to control their diet for 2 weeks before the study (run-in period). No additional antioxidants like vitamins C, E or coenzyme Q or excess of chocolate, red wine and beer were consumed. Drinking a cup of green tea, 2 dl red wine or 0.5 l beer daily was allowed. After that polyphenols administration began (week 0). Volunteers consumed 1 tablet of polyphenolic extract (100 mg) three times a day during 4 weeks (intervention period, week 4) followed by 2 weeks washout period in which tablets were not administrated (week 6).

Level of thiol was measured at the beginning (week 0), on fourth week and sixth week. After clinical investigation (blood pressure, weight, height, body mass index), the biological samples were taken (blood and urine). Baseline characteristics of volunteers are shown in Table 1. During the study baseline characteristics of volunteers were not significantly changed.

Fasting blood samples were collected into vacuum tubes containing ethylene diamine tetraacetic acid (EDTA) as an anticoagulant (1.6 mg EDTA/ml blood) and without an anticoagulant. The samples (serum and plasma) were centrifuged according to the standard protocol (2500 × *g* for 5 minutes). After that, they were aliquoted and stored at –80°C until use.

Determination of PON1 activities toward two substrates

Arylesterase activity of PON1-A: For the determination of PON1 arylesterase activity in serum, phenyl acetate was used as a synthetic substrate. Arylesterase activity was determined according to Gan et al. (1991). Interassay coefficient of variation is 7.1% and intraassay coefficient of variation is 4.2%.

Lactonase activity of PON1-L: For the measurement of PON1 lactonase activity in serum, dihydrocoumarin was used as a synthetic substrate. Lactonase activity was determined according to Aviram and Rosenblat (2008). Interassay coefficient of variation is 9.5% and intraassay coefficient of variation is 2%.

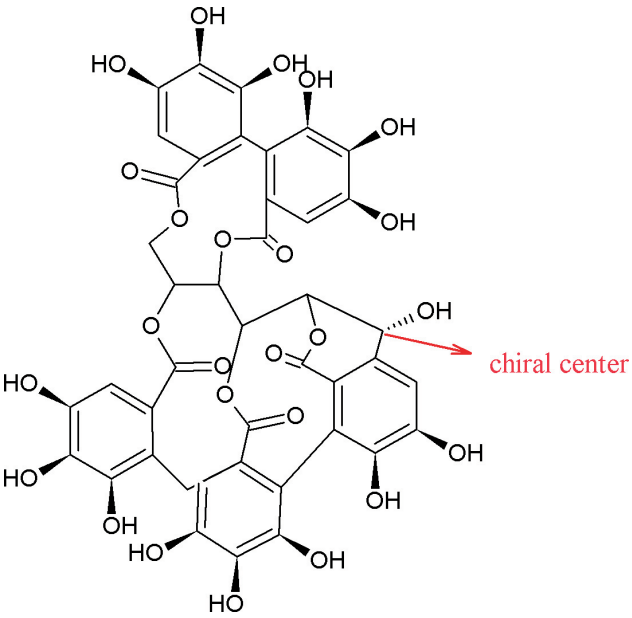

Figure 1. Castalagin as a (33beta)-isomer of vescalagin.

Determination of thiol samples

Samples for analyzing of thiols (Cys, HCy and GSH) concentration in plasma by HPLC system were pre-treated according to Garaiova et al. (2013). A mixture of 100 µl of plasma, 50 µl internal standards and 40 µl sodium borohydride was incubated for 30 minutes at 50°C to reduce and release protein-bound thiols, after which 100 µl perchloric acid was added to deproteination. After the sample centrifugation for 5 minutes at 14000 × *g* the supernatant was diluted 1:1 with the mobile phase. 20 µl of this mixture was injected into a column.

The chromatographic system consisted of an isocratic pump (DeltaChrom SDS 030, Watrex, Praha, Czech Republic) and automatic injector (Autosampler Basic-Marathon type 816, Spark Holland, Emmen, Netherlands). Separation

Table 1. Baseline characteristics of volunteers

	Age (years)	Height (m)	Weight (kg)	BMI (kg/m^2)
All	54.2 ± 6.5	1.70 ± 0.09	82.9 ± 19.4	28.4 ± 5.1
F (*n* = 12)	53.0 ± 7.7	1.64 ± 0.05	74.2 ± 16.9	27.4 ± 5.4
M (*n* = 8)	55.9 ± 4.3	1.79 ± 0.07	95.8 ± 15.8	29.9 ± 4.4

Data are presented as mean ± SD. *n*, number of volunteers; F, female; M, male; BMI, body mass index.

was performed on Purosher RP-18 250-4 (5 μm) (Merck, Darmstadt, Germany). The mobile phase (0.8 ml/min) was composed of 50 mmol/l phosphate buffer, 1 mmol/l octanesulfonic acid (pH 2.7) and acetonitrile (94:6 v/v) mixture. Aminothiols were detected by electrochemical detection (Coulochem II, ESA, Chelmsford, UK) composed of guard cell Model 5020 and analytical cell Model 5010A (ESA, Chelmsford, UK). DL-penicillamine was used as an internal standard. The response of amino acids was compared to response of internal standard. All samples were measured in triplicates.

Statistical analysis

Data were analyzed using StatsDirect statistical software (http://www.statsdirect.com, England: StatsDirect Ltd. 2008) and MS Excel 2010. Difference among glutathione and homocysteine at different time points where analyzed by Friedman test followed by all pairwise comparisons due to Conover. Correlation between two continuous variables was assessed using Pearson's correlation coefficient *r*. Simultaneous effect of age and gender on effect of treatment (difference between level of measured markers at week 0 and week 4) was analysed using multiple linear regression. All analyses were performed at level of significance 5%. The limits of detection (LOD) and quantification (LOQ) were defined as the concentrations for which the signal-to-noise (S/N) ratios were 3 and 10, respectively.

Results

In this work, the influence of Robuvit® on HCy, Cys and GSH total levels was considered. Their concentrations were monitored after run-in period (week 0), after 4 weeks Robuvit® intake (week 4) and after 2 weeks washout period (week 6). After 4 weeks of extract intake, the French oak wood extract had no significant influence on GSH levels ($p = 0.382$). On the other hand, Cys ($p < 0.001$) and HCy ($p = 0.029$) levels were significantly decreased (Table 2). No significant effect of gender was reported for all monitored amino acids. The level of HCy in women was decreased by 2.4 μmol/l ($p = 0.055$) in comparison to men, where reduced HCy levels by 0.6 μmol/l ($p = 0.640$). This gender dependence remains inconclusive.

The level of HCy is dependent on age ($r = 0.52$, $p < 0.010$) and also the effect of treatment on the HCy level significantly depended on age (Figure 2) ($p = 0.009$). It means that older individuals are more sensitive to treatment with higher reduction of HCy level. During the washout period the levels of HCy, Cys and GSH significantly increased again ($p = 0.008$, $p < 0.001$ and $p = 0.002$, respectively) to almost initial levels (Table 2).

The calibration curves of amino acid standards indicated high linearity ($r^2 > 0.99$) (not shown). The LOD and LOQ at S/N of 3 and 10 for HCy, GSH and Cys were 0.166, 0.173 and 0.484 μmol/l and 0.553, 0.577 and 1.612 μmol/l, respectively.

Table 2. Comparison of differences in plasma thiol levels after run-in (week 0), intervention (week 4) and washout (week 6) period

		week 0	week 4	p [a]	week 6	p [b]
cysteine	all	305.6 ± 49.1	220.7 ± 34.5	< 0.001	276.1 ± 56.6	< 0.001
	F	311.1 ± 42.6	236.1 ± 28.0	< 0.001	287.7 ± 52.9	0.014
	M	297.3 ± 52.6	197.5 ± 31.4	< 0.001	258.7 ± 61.0	0.006
glutathione	all	17.0 ± 5.5	16.4 ± 5.0	n. s.	20.4 ± 3.6	0.002
	F	18.4 ± 5.3	17.7 ± 5.2	n. s.	20.3 ± 4.4	n. s.
	M	14.9 ± 5.2	14.4 ± 4.0	n. s.	20.7 ± 2.0	0.006
homocysteine	all	10.35 ± 4.3	8.43 ± 2.6	0.029	9.65 ± 2.9	0.004
	F	9.7 ± 4.3	7.3 ± 1.9	0.055	8.7 ± 2.4	0.03
	M	11.0 ± 4.6	9.9 ± 2.7	n. s.	10.7 ± 3.5	0.09
PON1-A	all	115.8 ± 30.3	116.7 ± 29.2	n. s.	116.8 ± 31.3	n. s.
	F	119.8 ± 25.4	119.7 ± 23.9	n. s.	119.0 ± 23.9	n. s.
	M	109.8 ± 37.7	112.1 ± 36.9	n. s.	113.4 ± 41.7	n. s.
PON1-L	all	12.3 ± 2.7	12.0 ± 2.7	n. s.	12.0 ± 2.6	n. s.
	F	12.8 ± 2.6	12.5 ± 2.6	n. s.	12.6 ± 2.2	n. s.
	M	11.4 ± 2.8	11.1 ± 2.7	n. s.	11.2 ± 3.0	n. s.

Thiols data (in μmol/l) are shown as an average of total concentrations ± SD. F, female ($n = 12$); M, male ($n = 8$); n, number of volunteers; n. s., not significant ($p > 0.050$); PON1-A, paraoxonase-1 towards phenylacetate as a substrate; PON1-L, paraoxonase-1 towards dihydrocoumarin as a substrate; PON1-A and PON1-L data are shown as an average of the activity (U/ml) ± SD. [a] week 0 *versus* week 4, [b] week 4 *versus* week 6.

We investigated activity of PON1 on its arylesterase and lactonase activities (Table 2). The extract administration had no significant influence on PON1-A and PON1-L activities in human serum.

Discussion

The influence of French oak wood extract on total thiol levels was monitored. The level of Cys significantly decreased. We can only hypothesize that Cys is rather converted to degraded products like taurine or sulfate than be involved as a precursor for GSH synthesis. We did not notice a significant change in GSH level during intervention period. HCy level were significantly decreased. Chen et al. (2012) describe in their work that HCy has an indirect effect on synthesis of asymmetric dimethylarginine (ADMA). HCy can inhibit the enzyme that metabolizes ADMA. It also increases synthesis of ADMA by activating the endoplasmic reticulum stress pathway (Perna et al. 2010). Both HCy and ADMA are thought to mediate their adverse vascular effects (van Guldener et al. 2007) and we assume that decrease HCy level has positive influence also on ADMA level (not shown).

Older volunteers were more sensitive to Robuvit®. No significant effect of gender was reported. On the other hand, the effect of treatment (defined as difference between values of homocysteine at week 0 and week 4) positively depended on age ($p = 0.009$) and tended to a better response among women ($p = 0.055$), however, this remains inconclusive.

There are no known human studies on the effect of ellagitannins on HCy level. Only Kannan and Quine (2011) found that pretreatment with the basic component of ellagitannins, ellagic acid, prevents isoproterenol-induced oxidative stress and decreased HCy level in myocardial infarction in rats. In a number of other human studies some positive effects were found on markers of oxidative stress (increased activity of PON1, reduced TBARP in serum, reduced 8-oxo-dG level) or on lipid profile (Aviram et al. 2000; Cerdá et al. 2004; Pantuck et al. 2006; Heber et al. 2007; Rock et al. 2008; Fuhrman et al. 2010; Larrosa et al. 2010; López-Uriarte et al. 2010). Positive effects were not observed after ellagic acid or ellagitanins supplementation on inflammatory markers (IL-6 and CRP) (Trombold et al. 2010) and on cholesterol level in subjects with metabolic syndrome (Casas-Agustench et al. 2010). Among natural substances containing other polyphenols belongs to example cranberry juice containing namely anthocyanidins, cyanidins, peonidins and quercetin. Cranberry juice consumption did not significantly alter GSH and HCy levels in comparison to placebo (Duthie et al. 2006).

Results showed that the oak wood extract Robuvit® administration had no significant influence on paraoxonase activities towards both, arylester and lactone substrates and GSH level as well. It is in contradiction with result of Rock et al. (2008) who found enhancement of PON1 arylesterase, paraoxonase and lactonase activities. Similarly, Aviram et al. (2000) observed increased PON1 arylesterase activity after pomegranate juice consumption (tannins and anthocyanins). We assumed that these differences could be caused by application of different polyphenols treatment. Although, we did not observe increased activity of PON1, negative correlation between PON1-L activity and HCy level was noticed after intervention period (Figure 3). This confirms

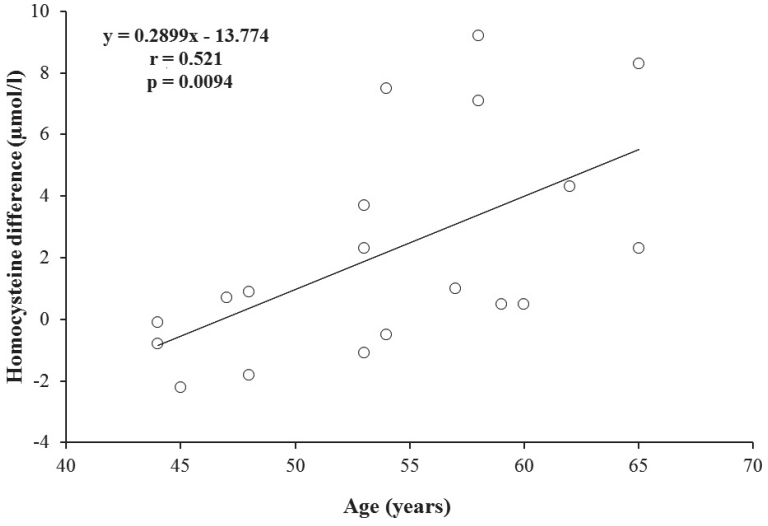

Figure 2. Regression analysis between polyphenols treatment (difference between homocysteine concentrations before and after intervention period) and volunteer´s age.

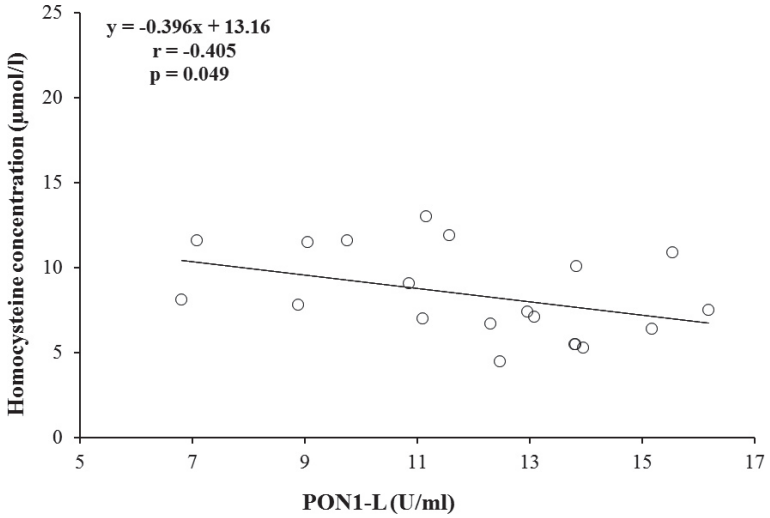

Figure 3. Correlation between homocysteine concentration and PON1-L activity after 4 weeks of polyphenols administration.

that PON1 might play an important role in HCy-thiolactone metabolism (Perla-Kaján and Jakubowski 2012).

Conclusion

Robuvit® had no significant influence on glutathione level and paraoxonase activities towards both, arylester and lactone substrates. On the other hand, homocysteine and cysteine levels decreased significantly.

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Conflict of interest. The authors declare that there are no conflicts of interest.

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