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# THE ESTROGENIC STATUS AND THE *COMT* GENOTYPE OF FEMALE BLOOD DONORS INFLUENCE THE PROTECTIVE ABILITY OF THE MEDITERRANEAN PLANT EXTRACTS AGAINST THE HYDROGEN PEROXIDE-INDUCED DNA DAMAGE IN LYMPHOCYTES

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The extent of oxidative DNA damage in lymphocytes can be used as a biomarker of the level of oxidative stress in the body. The comet assay has been widely used to measure such damage. The aim of our study was to evaluate: i) the extent of the oxidative DNA damage in lymphocytes isolated from blood of female donors taken in early and late follicular phases [low (LE) and high (HE) concentration of 17β-estradiol, respectively], ii) the susceptibility of these lymphocytes to hydrogen peroxide exposure, and iii) the protective ability of five plant extracts against the hydrogen peroxide-induced DNA damage. The effect of the catechol-*O*-methytransferase genotype (wild COMT H/H and mutated homozygote COMT L/L) of female donors was also analyzed. The amount of endogenous DNA damage was higher in HE lymphocytes as compared with LE ones, independently of the genotype. When lymphocytes were stratified by *COMT* genotype, the level of DNA damage was higher in L/L donors. The protective effect of pretreatment with plant extracts (1 and 10 μg/ml for 1 h) against the $H_2O_2$ (25 μM, 5 min. at 4°C)-induced oxidative DNA damage was observed only in H/H HE lymphocytes. In contrary, the plant extract pre-incubation enhanced the DNA damage in L/L HE lymphocytes. The plant extracts alone did not induce the DNA damage. The results showed that concentration of the circulating 17β-estradiol influenced the extent of endogenous oxidative DNA damage while the beneficial or hazardous effects of the plant extracts might depend on the *COMT* genotype and the estrogen level.

Key words: *oxidative DNA damage; comet assay; 17β-estradiol; catechol-O-methyltransferase; hydrogen peroxide; lymphocytes; plant extracts.*

## INTRODUCTION

Single cell gel electrophoresis seems to be the most frequently used method for determining the DNA damage. The original protocol was described by Ostling and Johanson (1) and has since undergone a number of modifications (2-4). The assay is sensitive enough to detect even endogenous DNA damage in cell (5,6). The method is also applied to detect DNA damage in human biomonitoring studies (7,8), in clinical radiobiology or in ecotoxicological studies (9-17). Another big advantage of this method, despite of its ability to detect single and double DNA strand breaks, is a possibility to assess a broad range of specific lesions recognized by the enzymes. These enzymes remove the altered bases and thus create the DNA break. Endonuclease III belongs to the class of enzymes, which recognize the oxidized pyrimidine bases and because of that has been commonly applied for the evaluation of the oxidative DNA damage in biomonitoring and intervention studies (18-24). The comet assay is also applied to monitor the extent of the DNA damage which occurs in lymphocytes and other cells after the estrogen treatment (25-27). Lymphocytes are also frequently used as model cells to evaluate the protective effect of plant extracts on the DNA damages induced by hydrogen peroxide, imitating the oxidative stress (28-30). However, the heterogenity of the levels of endogenous DNA damage evaluated in lymphocytes, derived even from the same blood donor, but withdrawn at different time, was observed (31,32). Furthermore, the extent of DNA damage in lymphocytes treated *ex vivo* can bring a different response depending on the phenotype of donors (12). Crott's study (33) was the first one, at least to our knowledge, in which the effect of genotype of lymphocyte donors was considered as a potential factor influencing the extent of DNA damage induced *in vitro*. Since the relationship between the genotype of blood donors, lifestyle, or nutritional status and endogenous DNA damage assessed in lymphocytes or whole blood samples was demonstrated already *in vivo* (33,34), it seems reasonable to expect such relation to occur also in *ex vivo* studies. We hypothesized that: i) the oxidative stress induced in the female body by the hormonal fluctuations occurring during the course of the natural menstrual cycle can be reflected by the oxidative DNA damage in lymphocytes, ii) the extent of this damage depends on the detoxifying ability of catechol-*O*-methyltransferase (COMT) encoded by the polymorphic *COMT* gene, and iii) the protective ability of plant extracts against the hydrogen peroxide-induced DNA damage *ex vivo* will be affected by such pre-determined physiological status of lymphocytes.

The COMT enzyme is involved in detoxification, through methylation, of catechol estrogens, the intermediates in 17β-estradiol metabolism, as well as of some polyphenols, the compounds widely distributed in the food of plant origin (36- 40). Their high concentrations and/or the prolonged exposure to both groups of catechol compounds can make them genotoxic, because if they are not rapidly methylated they undergo metabolic redox-cycling (38,41). In such situation the

corresponding quinone, semiquinone and also superoxide radicals are formed, causing oxidation of DNA bases and creating the ground for further mutations. The enzyme activity is controlled, in part, by a common genetic polymorphism. The COMT enzyme activity is 3-4 fold lower in the mutated variant (L/L) with a single nucleotide polymorphism which results from a Val108-Met substitution, as compared to the homozygous wild-type of *COMT* (H/H) (36). Such correlation between the *COMT* genotype and the differences in the activity of the enzyme was found in different human tissues. The genetic epidemiological studies which focus on the breast cancer are of a particular interest, as they investigate the COMT activities in relation to the hormone-related cancer risk (41-45).

Therefore, in our study, the healthy female blood donors in reproductive age, who did not take contraceptives were selected according to their catechol-*O*-methyltransferase (COMT) phenotype. The blood samples were drawn at early follicular phase of menstrual cycle (during the first three days) and at the late follicular phase (13-15 days after the first day of menstruation), when the 17β-estradiol concentration in plasma was several times higher as compared to early phase. The extent of oxidative DNA damage was measured in isolated lymphocytes by comet assay and the protective effect of the plant extracts against the DNA damage occurring during the *ex vivo* hydrogen peroxide treatment was evaluated.

## MATERIALS AND METHODS

### *Chemicals*

Agarose for electrophoresis grade ultra pure and agarose normal and low melting point ultra pure was purchased from Gibco BRL, UK; Agarose Top Vision, dNTPs (deoxynucleotides), Pfu DNA polymerase were purchased from Fermentas, Lithuania, NlaIII restriction enzyme was from Q-Biogen; DMSO (dimethyl sulfoxide), endonuclease III (generous gift from Dr. Barbara Tudek, IBB Warsaw.), ethidium bromide, propidium iodide, Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] were from Sigma, USA; DNA Extraction Kit was delivered by A&A, Poland; EDTA (ethylenediaminetetraacetic acid), Triton X100, supplied by MP Biomedicals; Primers were from IBB PAN, Warsaw, Poland; Salts and hydroxides were purchased from POC, Poland; the medium (RPMI 1640), serum (FBS), phosphate buffered saline (PBS) and antibiotics were from Gibco; microscope slides and cover slides came from DHN, Poland.

### *Plant extracts*

The plant samples from non-cultivated herbs, traditionally used for seasoning, were collected in southern Spain, Rio Segura Valley, Murcia (*Scandix australis, Thymus piperella*), southern Italy (*Oreganum heracleoticum*), and Crete (*Amaranthus sp*.); the extracts were obtained by ethanol extraction of dried plants, which were provided by the partners from the Local Food Nutraceuticals EU FP-5 project. The description of plants and the extraction procedure is reported in this Journal (paper by Rivera et al).

## *Study design*

This study is a part of the larger ongoing study of the flavonoids intake, *COMT* genotype and estrogen level in relation to oxidative DNA damage in pre-menopausal women (for which Dr. M. Kapiszewska is a Principal Investigator). Participants of the study were recruited from the cohort of female volunteers taking part in it. Female donors selected for the study comprised six homozygotes: COMT H/H (wild type) and COMT L/L (mutant variant). The subjects composed a very homogeneous group, as far as gender, age, weight, lifestyle and smoking habits were concerned. They did not take oral contraceptives or other hormonal medications within the previous six months. This allowed to minimize the impact of these confounding features. Subjects were 20-24 years old, with regular menstrual cycles of 28-33 days. They were not taking any other medications and they had no history of recent infections. The samples were collected from subjects in the early follicular phase (cycle day 1-3) and the late follicular phase (cycle days 13-15) designated in the text, Figures and Tables as LE and HE respectively.

The 17β-estradiol concentrations in plasma were determined by radioimmunoassay [using commercially available kits manufactured by Orion (Finland)] at the Department of Clinical Biochemistry, Jagiellonian University Hospital, Krakow, Poland.

The study protocol was reviewed and approved by the Jagiellonian University Medical College Ethics Committee.

## *Mononuclear blood cell preparation*

Experiments were performed on human peripheral blood lymphocytes isolated using the Histopaque technique according to the manufacturer's instructions and frozen at - 80°C at the $4x10^6$ cells/ml in the „freezing medium", consisting of 65% RPMI-1640, 25% fetal bovine serum and 10% DMSO.

## *Experimental procedure*

One hour before the experiments, the frozen lymphocytes were thawed, centrifuged, and, after washing in phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640, supplemented with 10% of fetal bovine serum and kept for 1 h at 37°C in a $CO_2$ humidified incubator, before the plant extracts at the final concentration of 1 or 10 μg/ml were added for 1 h. The stock solutions of the plant extracts in DMSO were diluted with culture media (RPMI 1640) to the appropriate final concentrations. The same concentration of DMSO was added to control samples.

Following pretreatment, lymphocytes were washed twice with PBS (to prevent direct extracellular interactions between compounds and hydrogen peroxide applied later), then the 85 μl of the cell suspension containing $2x10^4$ lymphocytes was added in 0.5% of low melting point agarose (LMPA) and spread on the microscope slides pre-coated with 100 μl of 0.5 % normal melting point agarose. After 2 min. of solidification on ice, the microscope slides were immersed in cold 25 μM hydrogen peroxide for 5 min. at 4°C. Then, following a quick rinsing in cold water, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 and 1 % Triton X-100) for 1 h.

After washing twice with Tris buffer (0.4 M, pH 7.5), once with enzyme buffer (40 mM HEPES/HCl, 1mM EDTA, 0.1 mg/ml BSA, pH 8) used for the endonuclease III dilution, the slides were covered with 50 μl of the endonuclease III (0.1 U/slide) for detection of oxidized pyrimidines and abasic sites (apurin/apyrimidine) and incubated for 45 min. at 37°C in humidified chamber. After rinsing twice with 0.4 M Tris, pH 7.5, the slides were placed side by side in a horizontal slab electrophoretic chamber (Kucharczyk, Poland), covered with freshly prepared electrophoresis buffer

(1 mM EDTA and 300 mM NaOH, pH > 13) and left for DNA unwinding for 40 min. at 4 °C. The electrophoresis was conducted at 0.74 V/cm for 30 min. The current was adjusted to 300 mA.

All steps after the start of cell lysis were done under dim light to prevent any induction of additional DNA damage. After electrophoresis, the slides were neutralized with Tris buffer (0.4 M, pH 7.5) and exposed to cold 100% methanol for 5 min. and allowed to dry. The slides were stored in a dry place until analysis (within a few days). Before viewing, the slides were placed in distilled water for 5 min. and stained with 2.5 μg/ml propidium iodide).

For visualization of the DNA damage, observations were made using a 10x objective (the final magnification was x 200) on an epifluorescence microscope (Olympus IX-50) equipped with appropriate filters. The microscope was linked to a computer through a CCD camera (i.CAM-hrM; sensor SONY ICX) for transferring images to the computer software. The image analysis system (Comet Plus from Theta System Gmbh, Germany) was used for the quantification of DNA damage.

The percentage of DNA in the tail (designated in the Figures as TDC) was automatically calculated. At least two slides per one subject, with 50 randomly selected cells per slide, were analyzed. Each experimental point was run in duplicate.

## *Viability Assay*

Fluorescein diacetate (10 μM) and ethidium bromide (25 μM) were used to determine cell viability. The cell viability, both in control samples and immediately after hydrogen peroxide treatment, was constantly found to be over 95 %.

## *COMT genotype analysis*

The catechol-O-methyltransferase polymorphism analysis was performed by PCR/RFLP method (3). Primers: up - 5'TACTGTGGCTACTCAGCTGTGC3', down - 5'GTGAACGTGGTGTGAACACACC3', reaction (performed with Pfu DNA polymerase, Fermentas): denaturation at 95°C for 4min., amplification: 30 cycles (95°C for 1 min.; 56°C for 30 sec; 72°C for 1 min.), extension: 10 min. at 72°C. PCR products were digested for 4 h at 37°C with 5U of NlaIII and separated on the 4% agarose gel. The lymphocytes from the wild-type homozygote female donors and those from mutated variant homozygotes were designated in the text, Figures and Tables as H/H and L/L, respectively.

## *Statistical analyses*

Statistical analyses were conducted using STATISTICA software, version 6. Data obtained from the comet assay (% tail DNA, „TDC") were analyzed by one-way ANOVA and post-hoc comparison of the means following log transformation due to heterogeneity of variance. Each of the measures were calculated for both the raw data and for data subjected to logarithmic transformation. A small constant (0.001) was added to the data prior calculations to circumvent the potential problem of taking the logarithm of 0. The data are presented as the Box & Whisker Plot. The P values, which indicate the post-hoc significance level for the respective pairs of means are displayed in the Tables. The comparison among the groups was made by one-way analysis of variance (Factorial ANOVA). The modifying ability of the plant extracts toward the extent of oxidative DNA damage induced by hydrogen peroxide was determined as follows: 100% - [TDC (plant extracts + $H_2O_2$)/TDC ($H_2O_2$)x100] and labeled on the plot axis as „an enhancement", if the calculated values were below 0, or „a protection", if the values were above 0.

The hypothesis of a lack of difference in the means of TDC parameters between two corresponding experimental groups was tested, namely between Group 1 - lymphocytes pretreated with plant extracts before hydrogen peroxide treatment, and Group 2 - lymphocytes treated by

hydrogen peroxide only. The P level was computed, based on the t-test to compare respective TDC values for each experiment. Differences were considered significant if $P<0.05$.

Non-parametric Mann-Whitney U-test was applied to determine the differences between the 17β-estradiol concentrations in four groups of lymphocytes (H/H HE, H/H LE, L/L HE, L/L LE).

## RESULTS

The aim of the study was to evaluate: i) whether the increase in the estradiol concentration during the follicular phase of menstrual cycle was reflected by the level of oxidized pyrimidine bases in lymphocytes, and ii) whether such a pre-determination could reveal differences in the susceptibility to hydrogen peroxide treatment and in the protective ability of the plant extracts evaluated by comet assay.

The concentrations of 17β estradiol in plasma was analyzed by radioimmunoassay. The mean values are outlined in Table 1 for six female donors stratified by phase of the menstrual cycle as well as the *COMT* genotype. The average plasma estradiol concentrations analyzed in early and late phases of follicular cycle were significantly different between both phases in both *COMT* variants: H/H LE *vs* H/H HE (P= 0.03) and L/L LE *vs* L/L HE (P=0.08). However, the concentrations were not statistically significantly different when compared between COMT H/H and COMT L/L donors at the same stage of follicular phase: H/H LE *vs* L/L LE (P = 0.93) and H/H HE *vs* L/L HE (P = 0.5). The wide variation was observed within L/L genotype group in late phase of follicular cycle. The difference in the estradiol concentrations between early and late follicular phases was twice greater in H/H donors, as compared to L/L donors.

The hypothesis, whether the concentrations of estradiol can influence the endogenous oxidative DNA damage independently of *COMT* genotype was tested. The results are presented in *Fig.1*. The extent of endogenous oxidative DNA damage in lymphocytes isolated during the late follicular phase, that is when lymphocytes were exposed to the higher concentration of estradiol (HE), was significantly higher (P=0.0002) as compared to the situation when they were isolated from the blood sample drawn in early follicular phase (LE).

Stratification of the investigated groups only by the *COMT* genotype revealed that the level of oxidative DNA damage was significantly higher (P = 0.012) in lymphocytes isolated from the COMT L/L, as compared with COMT H/H donors (*Fig. 2*).

The extent of oxidative DNA damage was comparable in all studied groups when lymphocytes were exposed to hydrogen peroxide (25 μM/ml, at 4°C, for 5 min.) (*Fig.1* and *2*).

When both factors (*COMT* genotype and 17β-estradiol) were considered in the same statistical analysis (Factorial ANOVA), the extent of DNA damage was significantly different between the following groups: H/H HE > H/H LE and L/L

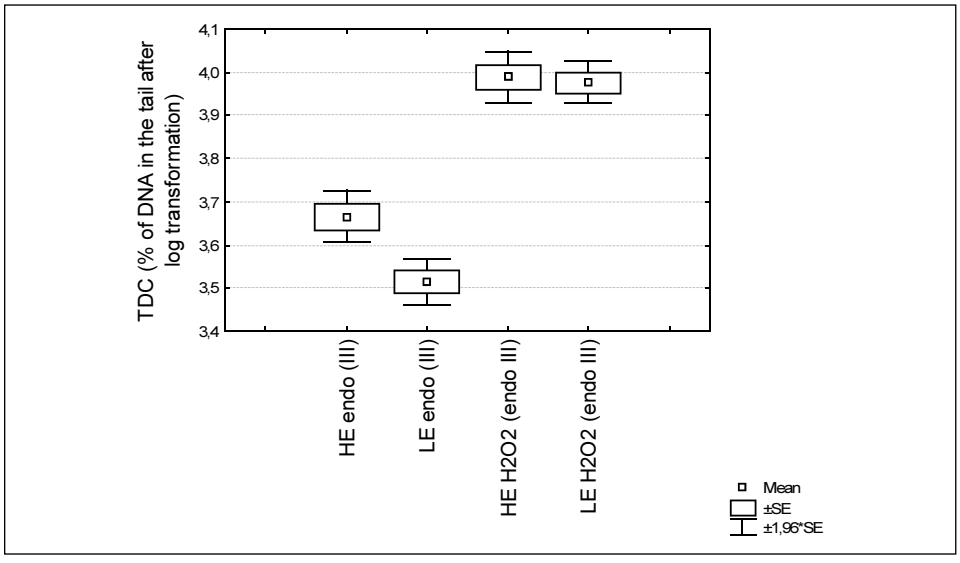


*Fig. 1* Box-and-Whisker plots showing a relationship between the extent of oxidative DNA damage, presented as the mean value of the percentage of DNA in the comet tail (after the logarithmic transformation) measured in lymphocytes isolated from the blood samples of female donors drawn: at early follicular phase of menstrual cycle - LE (during the first three days), and at the late follicular phase - HE (13-15 days after the first day of menstruation). The 17β-estradiol concentration in plasma was 21.6 pg/ml ± 5.8 and 151.5 pg/ml ± 27, respectively. These lymphocytes were treated by hydrogen peroxide (25μM, at 4°C for 5 min.) and then subjected to comet assay procedure. The results are presented also as the Box-and-Whisker plots. Statistical analysis was performed using one-way ANOVA and post-hoc comparison of the means.

LE > L/L HE (*Fig. 3*). In the next step, the effect of pre-treatment with four plant extracts (*Amaranthus sp., Origanum her., Scandix sp.* and *Thymus pi.*) on the hydrogen peroxide-induced DNA damage was assessed. Lymphocytes from the donors stratified according to the *COMT* genotype and estradiol level were incubated with plant extracts (1 or 10 μg/ml) for 1 h at 37°C and after the extracts removal, the cells were exposed to 25 μM hydrogen peroxide for 5 min. at 4°C. None of the tested plant extracts induced the oxidative DNA damage when given alone. The lack of effect of preincubation with the plant extracts on the hydrogen peroxide-induced DNA damage was observed in the groups with low estradiol level. The significant protection of the plant extracts was demonstrated only in H/H HE lymphocytes (*Fig. 4*). In particular, *Amaranthus sp.* and *Thymus pip.* at the concentrations 10 μg/ml, and *Origanum his.* at 1 and 10 μg/ml, showed the significant protective effect. Contrary to this observation, the significant enhancement of oxidative DNA damage induced by hydrogen peroxide was seen in lymphocytes with COMT mutated variant L/L (*Fig. 4*),

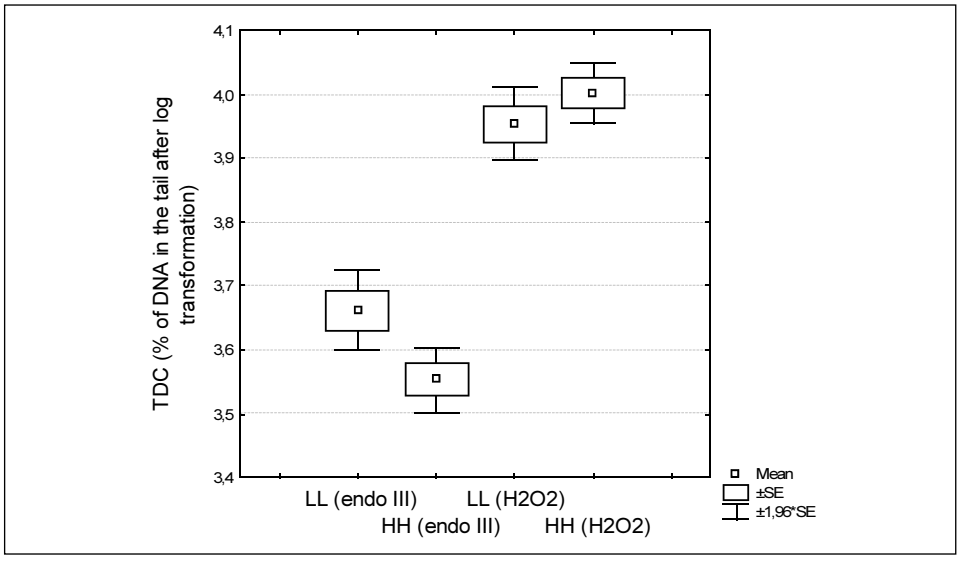


*Fig. 2* Box-and-Whisker plots showing a relationship between the extent of oxidative DNA damage presented as the mean value of the percentage of DNA in the comet tail (after the logarithmic transformation) measured in lymphocytes isolated from the blood samples of female donors (n=6) stratified by the *COMT* genotype: H/H (endoIII) - the wild group represent homozygote samples (*COMT H/H*), where both alleles contain a G at position 1947; LL - the mutated variant represent homozygote samples (*COMT L/L*), where both alleles contain an A at position 1947. These lymphocytes were treated by hydrogen peroxide (25μM, at 4°C for 5 min.) and then subjected to comet assay procedure. The results are presented also as the Box-and-Whisker plots. Statistical analysis were performed using one-way ANOVA and post-hoc comparison of the means.

preincubated with each plant extract, except for *Thymus pip.* at 10 μg/ml concentration.

## DISCUSSION

Lymphocytes and their DNA molecules are the most frequently chosen biological materials to study harmful effects resulting from metabolic changes (47), induced by drug administration or caused by the environmental toxins, the xenobiotics (48-51) and some nutritional deficiencies (33-35, 51-54). Their constant exposure to substances transported by the circulation make them very useful and reliable as a diagnostic tool. For example, the extent of the endogenous oxidative DNA damage analyzed in lymphocytes is used as a biomarker of the oxidative stress in the human body (54-57). Since lymphocytes are relatively easily available, they are often used in the epidemiological studies (5, 58-60). It was shown that the DNA in lymphocytes, as well as the comet assay used to study

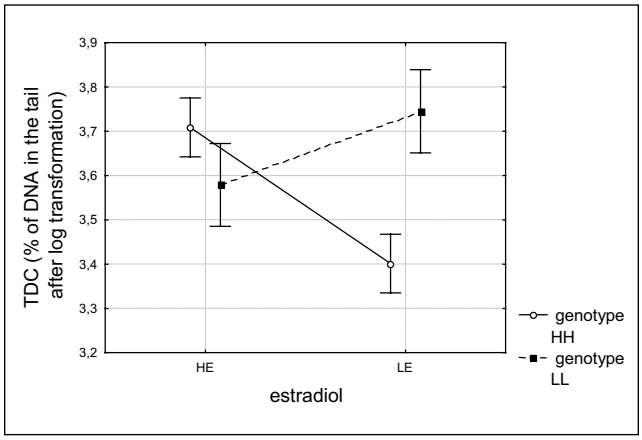


*Fig. 3* Relationship between the mean of oxidative DNA damage (after log transformation) in lymphocytes grouped according to the 17β-estradiol concentration in plasma - LE and HE (21.6 pg/ml ± 5.8 and 151.5 pg/ml ± 27, respectively), within genotypes of the *COMT* homozygotes: wild-type H/H and L/L - the mutated variant. The comparison of the groups was made by multivariate analysis of variance ANOVA/MANOVA.

the single/double breaks and the oxidative modification in the DNA bases, are sensitive enough to detect alterations in the antioxidant status in the body induced by the nutritional supplements or vitamins (5, 6, 61-63). Moreover, lymphocytes are commonly used to test the genotoxicity and the protective effect of different compounds or side effects of the drugs (28, 64-68). However, reasons of the observed inter-individual variations (69-72) between the extent of endogenous DNA damage in lymphocytes donated by the healthy donors need explanations. What especially should be taken into account is the influence of a single nucleotide polymorphism (SNP) in the genes, involved in the metabolism of the analyzed compounds, on the level of an endogenous DNA damage (12, 73). The association between the genetic polymorphism (MTHFR), nutrients, flavonoids and the endogenous DNA damage analyzed by comet assay was already demonstrated in human studies (34, 35). To our knowledge, the influence of SNP in genes, related to the metabolism of the particular nutrient, on the DNA damage was first investigated in *in vitro* studies by Crott et al. (33). They used the lymphocytes isolated from the donors with different methylenetetrahydroxyfolate reductase (MTHFR) polymorphism to study the extent of uracil misincorporation into DNA in relation to the folic acid concentrations in cell culture.

In our study, we attempted to analyze the involvement of the functional polymorphism of the gene, which encodes the enzyme influencing the redox cycling reactions and thus affects the amount of the free radicals, which damage DNA. Therefore, we analyzed the influence of the potentially harmful oxidized metabolites of estrogen on the extent of endogenous oxidative DNA damage in lymphocytes isolated from the female blood donors, differing in the 17β-estradiol concentration in plasma. The oxidative DNA damage can occur due to autoxidation of catechol estrogens and the catechol estrogen-semiquinone redox cycling, whereby the radicals and reactive oxygen species are generated. These reactions can result in enhancement of the single/double DNA strand breaks as

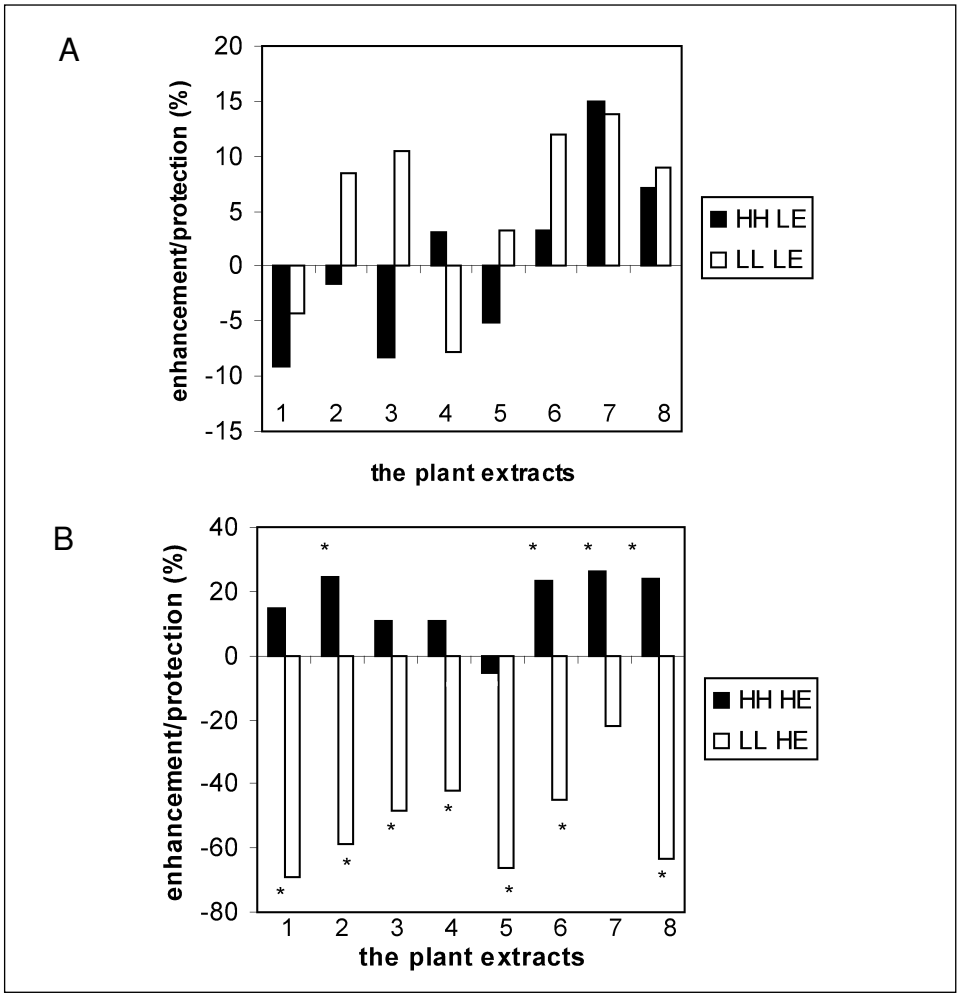


*Fig. 4.* The effect of the pre-incubation of lymphocytes with the plant extracts for 1 h on the hydrogen peroxide-induced oxidative DNA damage (25 μM, at 4°C for 5 min.). The following plant extracts were used: 1 and 2 *Amaranthus sp.*, 3 and 4 *Scandix australis*, 5 and 6 *Thymus piperella*, and 7 and 8 *Origanum heracleoticum* at the concentrations 1 or 10 μg/ml, respectively. The female donors (n=6) were stratified by the *COMT* genotype. The homozygote *COMT* wild-type is described as H/H and the homozygote *COMT* mutated variant as L/L. The lymphocytes were isolated from the blood drawn at the early follicular phase (*Fig. 4A*) - described as LE (the average 17β-estradiol in blood plasma is 17.5 pg/ml ± 9.3 for H/H and 25.7 pg/ml ± 6.4 for L/L), and at the late follicular phase (*Fig 4B*) - described as HE (the average 17β-estradiol in blood plasma is 224.5 pg/ml ± 77.7 for H/H and 170.5 pg/ml ± 172.8 for L/L). The modifying ability of the plant extracts toward the extent of oxidative DNA damage induced by hydrogen peroxide was determined as follows: 100% - [TDC (plant extracts + $H_2O_2$)/TDC($H_2O_2$)*100], and labeled on the Y axis as „an enhancement", if the calculated values were below zero, or „a protection" if the values were above zero. The enhancement or protection ability marked with * means that it is significantly different from the extent of oxidative DNA damage induced by the hydrogen peroxide treatment alone ($P < 0.05$).

well as in oxidative modification of purine and pirymidine bases (25,74,75). They can be blocked *via* several detoxication pathways, including *O*-methylation of catechol estrogens by catechol-*O*-methyl transferase (COMT) (35-37,76,77). The COMT is polymorphic in human population, which results in differences in enzyme activity and influences the capability of detoxification of catechol estrogens (41,77). A Val108 Met polymorphism in this gene is responsible for the 3- to 4-fold lower COMT activity as compared with the wild-type genotype (35,36,78,79). Thus, the amount of catechol estrogens in the circulation depends not only on their production but also on an efficiency of their inactivation. Because of that the donors of the blood samples were grouped according to their *COMT* genotype. They were described in this study as L/L (mutated variant homozygote) and H/H, (wild-type homozygote), respectively. We hypothesized that H/H donors of lymphocytes have higher ability to detoxify the catechol estrogens than the donors with the lower activity (L/L) and because of that, their lymphocytes should demonstrate the lower extent of oxidative DNA damage. The use of lymphocytes in such studies is justified by the fact that the estrogen receptors were identified in the majority of immune cells (80,81) and, in addition, the modulation of the immune system by female sex steroids in the menstrual cycle was also shown (82,83). To differentiate the exposure of lymphocytes to the estrogen concentration in plasma, the blood samples were drawn during the first three days of menstrual cycle, when the 17β-estradiol concentration was several times lower than during the late stage of the follicular phase (*Table 1*). If the estrogen level influences the extent of endogenous oxidative DNA damage in lymphocytes, the similar difference should be visible in the level of the DNA damage in lymphocytes. The obtained results confirmed our hypothesis, namely, that the significantly greater endogenous oxidative DNA damage occurred in the HE group, as compared with the LE group (*Fig. 1*).

The differences in the estradiol level between these two follicular stages were twice larger in H/H (224.5/17.5 pg/ml = 12.9; *Table 1*), as compared to L/L genotype (170.5/25.7 pg/ml = 6.6). These differences were also reflected in the oxidative DNA damage (*Fig. 2*) showing that the *COMT* genotype has more

*Table 1:* The mean values of the 17β-estradiol concentrations in plasma within the group of female blood sample donors (n=6) with *COMT* genotype: homozygote wild-type (H/H) and mutated variant (L/L). The blood samples were taken at early follicular phase of menstrual cycle - LE (during the first three days), and at the late follicular phase - HE (13-15 days after the first day of menstruation).

| COMT/ estradiol<br>N = 6 | pg/ml | SD |
|---|---|---|
| HH/ LE | 17.5 | 9.3 |
| HH/ HE | 224.5 | 77.7 |
| LL / LE | 25.7 | 6.4 |
| LL / HE | 170.5 | 172.8 |

impact on the extent of oxidative DNA damage (P= 0.009) than the 17β-estradiol concentrations in plasma (P=0.08) (*Fig.3*). As concentration of catechol estrogens is very low in humans, other factors may play a significant role in damaging the DNA. Since lymphocytes express the receptors for estrogens and also synthesize the active steroids locally, the interplay between hormones and the immune response can manifest itself as differences in the endogenous oxidative DNA damage. It has been postulated that estrogen in follicular phase contributes to development of the immune response classified as Th1, which is characterized by the production of cytokines, such as IFN-γ, IL-2, and TNF-β. These inflammatory mediators and reactive oxidants are generated dependently on estrogen concentration, leading to DNA damage, including strand breakage (84-88). Thus, the bigger extent of endogenous oxidative DNA damage in lymphocytes from late follicular phase, as compared with the early phase, does not necessarily reflect the harmful effect of estradiol but can be a sign of activation of Th1 cells, sometimes called „type 1 immunity".

The significantly greater DNA damage was also seen when the investigated groups of lymphocytes were stratified by the *COMT* genotype. The H/H HE lymphocytes showed larger DNA damage than the H/H LE lymphocytes, in contrast to the results obtained in L/L genotype (*Fig. 3*). The analysis of multivariants (*Fig. 3*) belonging to four groups of lymphocytes: H/H HE, H/H LE, L/L HE and L/L LE disclosed much more complex interactions between the analyzed estradiol concentration and *COMT* genotype. The wide variation of the mean of the 17β-estradiol concentrations within the L/L HE group and much smaller difference in the estrogen concentrations between HE and LE within L/L groups, may influence the DNA damage outcome. In such a situation, the results should be interpreted with a special caution. However, when the lymphocytes were grouped only according to the *COMT* genotype, the significantly higher level of endogenous oxidative DNA damage was seen in L/L lymphocytes, as compared with the H/H group (*Fig. 2*).

As already pointed out, the amount of the electrophilic reactive estrogen metabolites, such as quinone and semiquinone intermediates and reactive oxygen species, produced during the oxidative metabolism of estrogens, may be lowered by a sufficient activity of COMT, which can take place in the body of the H/H lymphocyte donors. It was shown that in the presence of a COMT inhibitor, the increased oxidative DNA damage was detected in cells *in vitro* when they were exposed to estradiol (77).

Since the rate of the COMT-catalyzed detoxification depends also on competition between the catechol estrogens and phytochemicals, in particular those which possess the catechol groups (89), it was interesting to evaluate the influence of pre-incubation with the plant extracts on the extent of oxidative DNA damage induced by hydrogen peroxide in each group of lymphocytes. None of the used plant extracts exerted any effect on LE lymphocytes, contrary to lymphocytes isolated from the blood drawn in late follicular phase (HE) (*Fig.4*).

One of the possible explanation of the observed phenomenon is that additionally to higher level of harmful cytokine in HE, as compared to LE (85-88), the presence of quercetin in the investigated plant extracts may enhance the secretion of the INF-gamma, as was already shown by Nair et al (90), leading to the accumulation of oxidative damage in DNA. Such an enhancement in oxidative DNA damage was observed only in lymphocytes (HE) from COMT L/L donors. It can be suggested that the high estrogen concentration in the presence of flavonoids from plant extracts may exceed the capacity of COMT to effectively catalyze their O-methylation into inactive metabolites. The resulting accumulation of catechol estrogens, the overloaded unmethylated flavonoids and pro-inflammatory cytokines may contribute to the observed DNA damage. The rapid and sufficient methylation of polyphenols, catalyzed by the high COMT activity in H/H lymphocytes, should prevent these reactions. The assumption was confirmed by the data, which demonstrated that lymphocytes from COMT H/H donors were significantly protected against the hydrogen peroxide-induced DNA damage by *Origanum heracleoticum, Amaranthus sp.* and *Thymus pip.* at the 10 μg/ml concentration. The only exception was *Scolymus au.*, which also was the only of the used plant extracts which exerted strong, more than 70%, inhibition of the IL-1-induced activation of the nuclear transcription factors NF kappaB and AP-1 (91).

It has to be noticed that not only the COMT activity was detected in lymphocytes (92-94), but also the enzymes involved in the estrogen synthesis in females were shown to be present in peripheral tissue, including the T lymphocytes (80-83). The biological activity, and especially the antioxidant, anticarcinogenic, cadrioprotective and estrogenic effects of the plant extracts and flavonoids, were widely studied (95-97). Recently, a renewed interest in flavonoids was fueled by the new research data showing that flavonoids, even at very low concentrations, can have a great impact on the regulation of gene expression (98-100) as well as on the cell-mediated immunity (101, 102).

The varying degrees of protective ability of four studied plant extracts could result from the nature of the active compounds, which contributed to their overall activity. The identification of their exact nature needs further study. It has to be also pointed out that all studied plant extracts showed the protective ability against oxidative DNA damage generated by hydrogen peroxide in lymphocytes isolated from male blood donors (COMT polymorphism was not investigated).

Taken together, these results indicate that the modulatory effect of a plant extract can depend on the physiology and genomic pattern of lymphocytes. Moreover, they also demonstrate that comet assay is an extremely sensitive tool to study the internal physiological changes that result in the DNA damage and confirm the usefulness of the comet assay in testing the protective ability of the plant extracts.

These data should be extrapolated to the human body very carefully. The amount of polyphenols in plasma, even after the high-flavonoid diet, rarely

reaches the concentrations used in this study. Moreover, the metabolic changes in polyphenols during digestion may also drastically affect their bioactivity. Nevertheless, since in many countries there is a growing interest in and the increased consumption of various food supplements, especially those containing plant extracts, the question can be raised regarding their impact on health. It is possible, as was demonstrated in our study, that the benefits and safety of flavonoid supplements can depend on the time of exposure, the interaction with other metabolites, and inherent genetic susceptibility of individuals.

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