MODIFYING EFFECT OF VITAMIN E AND ETHANOL ON BENZO[a]PYRENE INDUCED CHROMOSOME DAMAGE IN MICE BONE MARROW CELLS

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Summary. In the present study we have evaluated the potential of vitamin E protective effect against benzo[a]pyrene (B[a]P), induced clastogenicity in male C57BL x CBA mice bone marrow cells *in vivo*. Vitamin E (250 mg/kg b.w.) was given by gavage for 7 days prior to the administration of B[a]P (45 mg/kg b.w). For ethanol treatment animals were aloud to drink ethanol (10% water solution) *ad libitum* for 7 consecutive days. The animals were sacrificed by cervical dislocation 24 h after the last dose administration. The frequencies of chromosome aberrations were estimated in 100 metaphases per animal. Each group consisted of six animals. Chromosome aberration analysis revealed significant protective effect of vitamin E against B[a]P induced chromosome damage. Chromosome aberration frequency reduced significantly in animals co-treated with B[a]P and vitamin E as compared with those treated with B[a]P alone (2.00±0.26 *vs.* 9.50±0.72, P<0.0001). The protective effect of vitamin E in B[a]P + ethanol co-treated animals was lower when compared with B[a]P treated animals (2.80 ± 0.58 *vs*. 2.00 ± 0.26, P>0.05). Our results confirm the ability of vitamin E to reduce the chromosome damage induced by benzo[a]pyrene in mice *in vivo*. Treatment with ethanol had no significant effect on the frequency of chromosome aberrations under conditions of the current study.

Keywords: vitamin E; benzo[a]pyrene; ethanol; chromosome aberration; bone marrow; mice.

MODIFIKUOJAMASIS VITAMINO E IR ETANOLIO POVEIKIS BENZO[A]PIRENU INDUKUOTŲ CHROMOSOMŲ PAŽAIDŲ DAŽNIUI PELIŲ KAULŲ ČIULPŲ LĄSTELĖSE

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Santrauka. Tirtos vitamino E antiklastogeninio poveikio galimybės sumažinant benzo[a]pirenu (B[a]P) indukuotų chromosomų pažaidų dažnius C57BLxCBA pelių (♂) kaulų čiulpuose. Vitaminu E (250 mg/kg k. sv.) pelės buvo veikiamos zonduojant į skrandį 7 dienas iki poveikio B[a]P (45 mg/kg). Etanolis (10 proc.) duotas *ad libitum* 7 dienas vietoje geriamojo vandens. Gyvūnai numarinti taikant stuburo dislokaciją praėjus 24 val. nuo poveikio paskutine doze. Chromosomų aberacijos analizuotos 100 metafazinių plokštelių, gautų iš kiekvieno gyvūno. Kiekvienoje tiriamojoje grupėje buvo 6 gyvūnai. Nustatytas stiprus antiklastogeninis vitamino E poveikis. Chromosomų aberacijų dažnis vitaminu E ir B[a]P veiktoje gyvūnų grupėje buvo statistiškai reikšmingai mažesnis palyginti su gyvūnais, veiktais tik B[a]P (2,00±0,26 *vs.* 9,50±0,72; p<0,0001). Apsauginis vitamino E poveikis buvo neženkliai mažesnis grupėje gyvūnų, veiktų B[a]P, vitaminu E ir etanoliu, palyginti su gyvūnais, veiktais B[a]P ir vitaminu E (2,80 ± 0,58 *vs*. 2,00 ± 0,26; p>0,05). Tyrimų rezultatai patvirtina vitamino E veiksmingumą mažinant benzo[a]pirenu indukuotų chromosomų pažaidų dažnius *in vivo*. Poveikis etanoliu reikšmingos įtakos chromosomų pažaidų dažniui neturėjo.

Raktažodžiai: vitaminas E, benzo[a]pirenas, etanolis, chromosomų aeracijos, kaulų čiulpai, pelės.

Introduction. Polycyclic aromatic hydrocarbons (PAHs) comprise an important class of environmental genotoxins, benzo[a]pyrene (B[a]P) being the most well studied PAH. B[a]P is ubiquitously distributed throughout the environment and like other PAHs is formed during incomplete combustion of organic material and high temperature processing of crude oil, coal, coke, or other industrial carbon compounds. The major anthropogenic sources of B[a]P include combustion of fossil fuels, coke oven emissions and vehicle exhausts, while the main natural sources are volcanic eruptions, forest and peat fires. In ambient air, B[a]P is predominantly associated with fine particulates but also appears as gaseous vapour. B[a]P and other PAHs are also find in cigarette smoke, in smoked and grilled food (Shi et al., 2010). Thus, all living organisms including humans may be exposed to B[a]P from air, water and food through inhalation, ingestion and skin contact.

It is well documented that exposure to B[a]P may cause adverse health effects. Animal studies have shown that B[a]P can induce a number of toxic effects, including reproductive system and bone marrow toxicity, adverse respiratory effects, immunosuppression, genotoxicity, carcinogenicity. Carcinogenic effects of B[a]P have been demonstrated in a variety of laboratory animal species such as mice, rats, hamsters, rabbits, ducks, monkeys. Tumours in different target sites (bone marrow, lymphoid organs, testes) have been produced in test animals exposed by different routes of administration (inhalation, dermal exposure, intraperitoneal injection, given in the diet). Human epidemiological studies have shown that occupational exposure to B[a]P increases the risk of lung, stomach, bladder, skin cancers (IARC monographs, 2010).

Genotoxic effects of B[a]P have been demonstrated in both *in vivo* tests in rodents and occupationally exposed workers and *in vitro* tests using mammalian (including human) cell lines, as well as in prokaryotes (Gajecka et al., 1999; Frankič et al., 2008). The International Agency for Research on Cancer (IARC) considers B[a]P a human carcinogen (Group 1) (IARC monographs, 2010).

B[a]P requires metabolic activation to exhibit its carcinogenic and mutagenic activities. The major activation pathway of B[a]P is considered to be the formation of diol-epoxides, including B[a]P-7,8-diol-9,10-epoxydes, which are identified to be the ultimate carcinogenic and mutagenic metabolites (DNA-reactive metabolites). Diol epoxides can form stable and depurinating adducts with DNA through electrophilic carbonium ions (Xue and Warshawsky, 2005). Recent studies showed that human cytochrome P450 enzymes CYP1A1, 1A2, and 1B1 have key roles in activating B[a]P and other PAHs to their diol-epoxides (Shimada and Fujii-Kuriyama, 2004; Shi et al, 2010). B[a]P metabolism is also associated with the production of reactive oxygen species (ROS) and induction of oxidative stress that could be involved in B[a]P-induced oxidative damage to DNA, its genotoxicity and carcinogenicity (Briede et al., 2004; Shimada, 2006; Hanzalova et al., 2010).

There are reports that various factors (including ethanol) may modify CYP activities and consequently influence B[a]P metabolism, its toxicity and carcinogenicity. It is well documented that though ethanol *per se* is considered to be neither carcinogenic, mutagenic nor genotoxic, it induces an increase in lipid peroxidation and increases the clastogenic potential of many mutagens (including cigarette smoke condensate) (Philips and Jenkinson, 2001; Sutandyo, 2010).

Vitamin E is considered to be one of the most important antioxidants to prevent oxidative injury of DNA and other cell structures (Brigelius-Flohe and Traber, 1999). Vitamin E is the collective name for eight different tocopherols (d-α-, d-β-, d-γ- and d-δ-tocopherols) and tocotrienols (d-α-, d-β-, d-γ- and d-δ-tocotrienols), with d-α-tocopherol being the most biologically active form. Vitamin E is known to scavenge peroxy radicals, singlet oxygen and superoxide radicals and thus protect DNA from free radicals attack (Bisby et al., 1996; Brigelius-Flohe, 2009).

The aim of the present study was to study the potential protective effects of vitamin E toward clastogenic action of benzo[a]pyrene in mice bone marrow cells. In addition, modulatory effects of chronic ethanol consumption on benzo[a]pyrene induced clastogenicity were studied.

Materials and methods. Chemicals: vitamin E (d-α-tocopherol acetate, CAS No. 58-95-7), benzo[a]pyrene (97% purity, CAS No. 50-32-8), colchicine (95% purity, CAS No. 64-86-8), Giemsa stains were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Cyclophosphamide was used in commercial form 'Endoxan' (ASTA Medica AG, Germany).

Animals and treatment schedule: male C57BL x CBA mice 10-12 weeks old and having a body weight of 20-25 g were used in the study. The animals were supplied from the Animal Facility of Institute of Immunology, Vilnius University (now State Research Institute Center for Innovative Medicine). Experiment conditions were complying with good laboratory practices and with the Law of the Republic of Lithuania on the Care, Keeping and Use of Animals as well as secondary legislation - Order of the State Food and Veterinary Service of the Republic of Lithuania "On Veterinary Regulations on Breeding, Handling and Transportation of Laboratory Animals" and "On the Use of Laboratory Animals in Scientific Experiments" (Law of the Care, Welfare and Use of Animals, 2002).

The animals were acclimatized for one week before the study; they were housed under constant conditions of temperature, humidity and a light/dark cycle (12 h/12 h). Commercial pellet diet and fresh drinking water were provided *ad libitum.* The animals were housed 6 mice per cage and randomly divided into 10 groups:

1. C – control group (untreated animals).
2. VH – vehicle-treated group, negative control; received 0.1 ml olive oil by gavage once a day for 7 consecutive days.
3. CP – positive control group; received a single dose of cyclophosphamide (20 mg/kg b.w.) by the intraperitoneal route.
4. VE – group; received vitamin E (250 mg/kg b.w.) by gavage once a day for 7 consecutive days.
5. BP – group; received a single dose of benzo[a]pyrene (45 mg/kg b.w. in 0.1 ml olive) by gavage.
6. VE+BP – group; received vitamin E (250 mg/kg b.w.) for 7 consecutive days and benzo[a]pyrene (45 mg/kg b.w. in 0.1 ml olive) just after the last dose of vitamin E on the 7th day.
7. ET – group; animals were aloud to drink ethanol (10% water solution) *ad libitum* for 7 consecutive days.
8. VE+ET – group; received vitamin E (250 mg/kg b.w.) for 7 consecutive days and were aloud to drink ethanol (10% water solution) *ad libitum*.
9. BP+ET – group; received single dose of benzo[a]pyrene (45 mg/kg b.w. in 0.1 ml olive) and were aloud to drink ethanol (10% water solution) *ad libitum.*
10. VE+BP+ET – group; received vitamin E (250 mg/kg b.w.) for 7 consecutive days, benzo[a]pyrene (45 mg/kg b.w. in 0.1 ml olive) just after the last dose of vi-

tamin E on the 7th day and were aloud to drink ethanol (10% water solution) *ad libitum*.

The animals were sacrificed by cervical dislocation 24 h after the last dose administration. Colchicine was injected intraperitoneally at the dose of 2 mg/kg b.w. 90 minutes before sacrifice.

Chromosome aberration assay: both femurs were dissected from each animal. Bone marrow was collected by washing the femurs with 0.55% KCl hypotonic solution, incubated for 25 min. at 37^0C and then centrifuged at 150 x *g* for 8 min. Cells were fixed in methanol-glacial acetic acid (3:1). Slides were prepared by a flame-drying procedure and then stained with 5% Giemsa. Slides were coded and scored blind by the single scorer at a magnification of 1000x (Nikon Eclipse E200). Only well-spread metaphases with 40±1 chromosomes were used for analysis. The frequencies of chromosome aberrations were estimated in 100 metaphases per animal. The types of chromosome aberrations included chromatid (ctb) and chromosome breaks (csb) and chromatid exchanges (cte). Aberrations were classified according to Savage's classification (1976). Gaps were counted but not included in the statistical analysis.

Statistical analysis: all data were processed with SPSS 12.0 statistical software. All values were expressed as mean ±S.E.M. of six animals per group. Data were analyzed using one-way analysis of variance (ANOVA) followed by Post Hoc LSD test for comparison between the various groups. Values were considered as significant if $P< 0.05$.

Results and discussion. The results of cytogenetic studies in mouse bone marrow cells are presented in Table 1.

Table1. **Frequencies of aberrant metaphases and chromosome aberrations in mouse bone marrow cells after treatment with benzo[a]pyrene, vitamin E and ethanol *in vivo***

Treatment group	Aberrant metaphases, % ± S.E.M.	Chromosome aberrations[a] /100 cells, mean ± S.E.M.		
		ctb	cte	csb
C	1.20 ± 0.37	0.6 0± 0.24	0.60 ± 0.24	0
VH	1.2 0 ± 0.37	1.00 ± 0.32	0.20 ± 0.20	0
CP	17.17 ± 1.25[b]	12.83 ±0.87	5.00 ±0.73	1.50 ± 0.43
VE	2.50 ± 0.43	1.17 ±0.31	1.33 ±0.21	0
BP	9.17 ±0.60[b]	6.00 ± 0.86	3.50 ± 0.78	0
VE + BP	2.00 ± 0.26[c]	1.33 ± 0.30	0.67 ± 0.10	0
ET	1.60 ± 0.24	1.20 ± 0.20	0.40 ± 0.40	0
VE+ET	1.80 ± 0.20	1.20± 0.37	0.60 ± 0.24	0
BP+ET	8.00±1.09[b]	5.20 ± 0.86	3.00 ± 0.44	0.20 ± 0.20
VE+BP+ET	2.80 ± 0.58[c,d]	1.60 ± 0.51	1.20 ± 0.37	0

Notes: [a]ctb, chromatid breaks; cte, chromatid exchanges; csb, chromosome breaks.
[b]Significant induction against the vehicle control (VH group) at P<0.0001.
[c]Significant protection against benzo[a]pyrene (BP group) at P<0.0001.
[d]P<0.05 when compared with the controls.

As expected, animals treated with cyclophosphamide (CP group) and benzo[a]pyrene (BP group) showed a high frequency of chromosome aberrations as well as abnormal metaphases when compared with the controls (C group). The most frequent chromosome aberrations observed were chromatid breaks. These results agree with those reported in other investigations, which showed that chromatid breaks are the changes most frequently induced by cyclophosphamide and B[a]P *in vivo* (Antunes and Takahashi, 1998). Various types of chromosome aberrations, including breaks, gaps, rings, dicentrics, were induced in Chinese hamster ovary (CHO) and Chinese hamster lung (Don) cells after treatment with B[a]P *in vitro* (Smalls and Patterson, 1982).

Exposure to vitamin E alone (VE group) did not induce any significant increase in the frequency of chromosome aberrations as compared with the vehicle-treated (VH group) and non-treated control (C group) animals, showing that this concentration of vitamins E is not clastogenic. Chromosome aberration analysis revealed significant protective effect of vitamin E against benzo[a]pyrene induced chromosome damage (Fig. 1).

Chromosome aberration frequency reduced significantly (up to the control level) in animals co-treated with benzo[a]pyrene and vitamin E as compared with those treated with benzo[a]pyrene alone (2.00±0.26 *vs.* 9.50±0.72, P<0.0001). The treatment with ethanol did not increase aberration frequency and significant reduction of aberrations was observed in B[a]P-vitaminE-ethanol co-treated animals when compared with animals treated with benzo[a]pyrene alone (2.80±0.58 *vs*. 9.50±0.72, P<0.0001). However, the level of chromosome aberrations in the co-treated group remained elevated and was significantly higher as compared with the controls (2.80±0.58 *vs.* 1.20±0.37, P<0.05).

Vitamin E is one of the most extensively studied antioxidants. The results obtained in previous studies demonstrate that vitamin E exerts antimutagenic effect against genotoxic effects induced by various chemical agents in different test subjects. Vitamin E was reported to reduce

the chromosome damage induced by carbon tetrachloride in ovine peripheral lymphocytes. Moreover, the improvement in proliferation was observed (Šivikova et al., 2001). Vitamin E has been demonstrated to significantly reduce the percentage of benzo[a]pyrene induced chromosomal aberrations *in vitro* in Chinese hamster ovary (CHO) and Chinese hamster lung (Don) cells (Smalls and Patterson, 1982). However, VE had no effect on the number of benzo[a]pyrene induced *his*$^+$ revertants in TA98 *S. typhimurium* strain, but significantly decreased the mutagenic activity of cigarette smoke in *S. typhimurium* TA98 (Balansky et al., 1994).

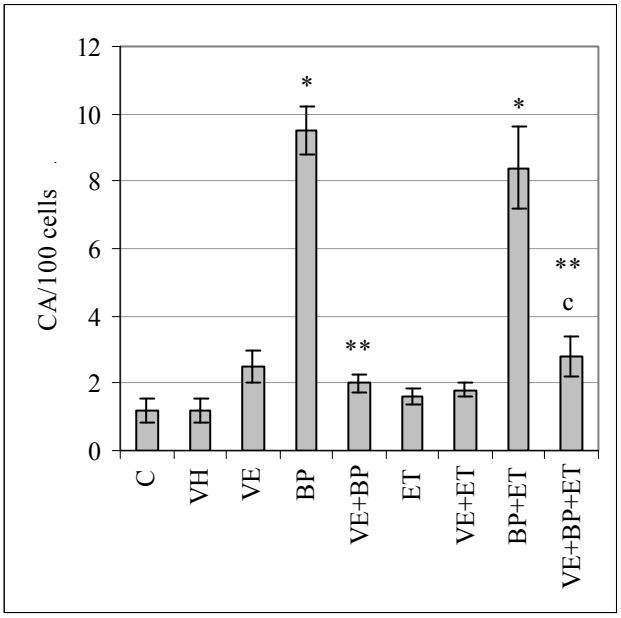

Fig. 1. **Protection against benzo[a]pyrene** (BP) **induced chromosomal aberrations** (CA) **by vitamin E** (VE) **in mice bone marrow cells *in vivo***

Notes: *Significant induction against the vehicle control (VH group) at P<0.0001.

**Significant protection against benzo[a]pyrene (BP group) at P<0.0001.

^{c}P<0.05 when compared with the controls.

ET – ethanol; C – control group.

The protective effect of vitamin E was demonstrated when vitamin E was applied simultaneously with or after the benzo[a]pyrene in human lymphocytes by the Comet assay. It is interesting to note that the background level of DNA damage in the presence of vitamin E was lower than in the system without vitamin E. The authors (Gajecka et al., 1999) assume that vitamin E protective activity is not connected with the steps in metabolic activation or DNA repair, but more likely vitamin E acts as competitor of DNA molecule in reaction with the reactive oxygen species. Our results confirm this assumption and the ability of vitamin E to reduce the chromosome damage induced by benzo[a]pyrene *in vivo*.

Many studies have been carried out on the toxicology of ethanol, the majority in the context of the effects of the consumption of alcohol in beverages. However, the available data on ethanol genotoxicity are incomplete and sometimes contradictory. There is clear evidence that ethanol is not clastogenic or mutagenic in bacterial or mammalian cells *in vitro*. No chromosome aberrations or micronucleus were found in human lymphocytes, HeLa cells, Chinese hamster cells treated with ethanol (reviewed in Philips and Jenkinson, 2001). Test for chromosome aberration and micronucleus induction *in vivo* are negative also with rear exceptions for micronucleus. However, there is some evidence that ethanol induces SCEs *in vivo* and can also act as aneugen at high doses. Though ethanol should not be classified as a mutagen, it has marked effect on the metabolism of the liver, affecting the metabolism of xenobiotics and increasing the levels of oxygen radicals (Yan et al., 2010). Ethanol administration induces an increase in lipid peroxidation either by enhancing the production of ROS and/or by decreasing the level of endogenous antioxidants. Ethanol was demonstrated to inhibit removal of benzo[a]pyrene diol-epoxide-DNA adducts in human mammary epithelial cell line MCF-10F, and this inhibition is considered to be related to ethanol-associated oxidative stress (Singletary et al., 2004; Yu et al., 2010).

It is documented that clastogenic potential of many mutagens (including cigarette smoke condensate) increased when ethanol was added concurrently with the mutagens in cell cultures *in vitro* (Sutandyo, 2010). In our study, ethanol even slightly decreased the frequency of chromosome aberrations after concurrent treatment of animals with benzo[a]pyrene when compared with the animals treated with benzo[a]pyrene alone (8.40±1.21 *vs.* 9.50±0.72, P>0.05). However, the protective effect of vitamin E in benzo[a]pyrene + ethanol co-treated animals was lower when compared with benzo[a]pyrene treated animals (2.80 ± 0.58 *vs*. 2.00 ± 0.26, P>0.05).

Conclusion. Our results indicate that vitamin E protects against the clastogenic effects of benzo[a]pyrene in mice *in vivo*. Treatment with ethanol had no significant effect on the frequency of chromosome aberrations under conditions of the current study.

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