Carvacrol given to rats in drinking water reduces the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2

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Carvacrol represents a very frequent constituent of essential oils and occurs in many kinds of plants. Though human beings comequite often into close contact with this phenol derivative, its biological effects are not sufficiently known. In this paper we investigated the influence of carvacrol given to rats in drinking water on resistance of their liver and testicular DNA against the oxidative agent hydrogen peroxide (H_2O_2). Carvacrol was dissolved in tap water and given to rats either in concentrations of 30 and 60 mg/1kg/day during 7 days or in concentrations of 15 and 30 mg/1kg/day during 14 days. Control animals were given tap water only. After the given time the rats were sacrificed and hepatocytes and testicular cells were isolated and treated with different concentrations of H_2O_2 (0-250 μM, 5 min, on ice). Then the level of DNA lesions was detected by single cell gel electrophoresis. The results of both types of application of carvacrol showed that DNA of cells isolated from carvacrol-treated animals was significantly more resistant to damaging effects of hydrogen peroxide than DNA of control animals. We assume that the observed DNA-protective effects of carvacrol, which was given to rats during a short time of their life, could be associated with an increase of antioxidant activity of liver and testicular cells in these animals.

*Key words: Carvacrol, hepatocytes, testicular cells, single cell gel electrophore*sis

Essential oils (EOs), as well as their purified or synthetized compounds, are used in perfumery, aromatherapy, cosmetics, for flavoring food and drink, and in health care. Carvacrol (cymophenol; $C_6H_3CH_3(OH)(C_3H_7)$ – 5-isopropyl-2-methyl phenol) is a constituent of the essential oil of *Origanum hirtum*, oil of thyme, oil obtained from pepperwort and wild bergamot and was found also in several other EOs. Carvacrol, which may be extracted from EOs or synthetically prepared, represents a thick oil liquid with melting point 0°C and boiling point 236–237°C. Oxidation with ferric chloride converts it into dicarvacrol. Carvacrol is isomeric with thymol (a monoterpene phenol derivative of cymene; $C_{10}H_{13}OH$). Both thymol and carvacrol can bind to major and minor grooves of B-DNA [1]. Spectroscopic evidence showed that thymol and carvacrol interaction with DNA occurred mainly through H-bonding of the thymol and carvacrol OH group to guanine N7, cytosine N3, and backbone phosphate group. Carvacrol, like many other components of EOs, have been shown to exhibit a strong antibacterial activity against a wide spectrum of bacteria [2] and an anticandidal activity, comparable with the anticandidal activity of the commercial preparation nystatin [3]. Antifungal activity is manifested by the EO of *Thymus pulegioides,* containing high amounts of carvacrol and thymol [4]. With respect to these activities, carvacrol and many other plant volatiles and their components have great potential for application in postharvest crop protection and food preservation. It is, however important to evaluate such compounds for mutagenic and antimutagenic properties. Stammati et al. [5] used several short-term microbial *in vitro* assays to evaluate genotoxicity of cinnamaldehyde, carvacrol, thymol and S(+)-carvone. At non-toxic doses, carvacrol and thymol increased the number of revertants in the Ames test by 1.5-1.7-times, regardless of metabolic activation. In the SOS-chromotest, none of the four plant volatiles caused DNA damage at non-toxic doses. In the DNA repair test, a marked dose-dependent differential toxicity was observed with carvone and, to a lesser extent, with cinnamaldehyde, while with thymol and carvacrol this effect was less pronounced. Cytogenetic analysis of human blood peripheral lymphocytes

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treated with carvacrol were carried out by Ipek et al. [6] using the *in vitro* sister chromatid exchange (SCE) assay. According to the data obtained, carvacrol did not increase the formation of SCE, while it inhibited the rate of SCE induced by mitomycin C and acted as an antigenotoxic agent. On the other side, Azirak and Rencuzogullari [7], who investigated the *in vivo* genotoxic effects of carvacrol and thymol on rat bone marrow cells, found that both carvacrol (10, 30, 50, and 70 mg/kg b.w.) and thymol (40, 60, 80, and 100 mg/kg b.w.) administered intraperitonally increased significantly the level of structural and total chromosome abberations (CA) for all treatment periods (6, 12, and 24 h) when compared with control.

This report comprises experiments aimed at DNA-damaging and DNA-protective effects of carvacrol on hepatocytes and testicular cells isolated of rats which consumed carvacrol in drinking water. DNA-protective effects of carvacrol were evaluated on the basis of the increased resistance of isolated hepatocytes and testicular cells against the oxidative agent hydrogen peroxide (H_2O_2). The connection between intake of carvacrol and response of hepatocytes and testicular cells to treatment with hydrogen peroxide was evaluated by single cell gel electrophoresis, which represents a sensitive method for measuring DNA damage at the level of single cells. The genotoxic effect of hydrogen peroxide is mediated mainly by the highly reactive oxidant ·OH, generated by the reaction of reduced transition metals (Fe^{2+} or Cu^{2+} ions) with H_2O_2 via Fenton reactions [8]. The DNA damage profile induced by ·OH was shown to consist of approximately equal levels of oxidized DNA bases, abasic sites, and strand breaks [9].

Material and Methods

Animals. Twelve sexually mature male Sprague-Dawley (SD) rats obtained from ANLAB, Czech Republic, with a mean body weight (b.w.) of 250 g (range 230-280 g) were used in two individual experiments. The animals were obtained from an in-house strain and were maintained in a room with 12 hr light/dark cycle, room temperature 22±2°C, relative humidity 55±5%. They were housed in plastic cages on hardwood bedding (1 rat per cage). A standard diet (containing nutriment, amino acids, biofactors, and minerals) was given. Food consumption for each cage was recorded daily and individual body weights were recorded weekly. The compound tested, carvacrol, was added to drinking water in different concentrations. Water (+/- carvacrol) was provided *ad libitum*. At the seven-day-long carvacrol treatment (experiment N°1), two parallel rats, which comprised a control group, drank tap water only and four animals drank carvacrol supplemented water (two parallel rats by 30 mg/day/kg b.w. and two parallel rats by 60 mg/day/kg b.w.). At the fourteen-day-long carvacrol treatment (experiment N°2), two parallel control animals drank tap water only and four animals drank carvacrol supplemented water (two parallel rats by 15 mg/day/kg b.w. and two parallel rats by 30 mg/day/kg b.w.). After 7/14 days, the total b.w. gain was ~ 40-80 g in both control and carvacrol drinking rats. No degeneration of inner organs or worsening of the overall health condition were observed in carvacrol supplemented rats. After 7/14 days the rats were ethically sacrificed by i.p. dose of thiopental and from each animal hepatocytes and testicular cells determined for further processing were isolated. The experiments were conducted under the guidelines of the Animal Ethics Committee and were approved by the State Veterinary and Food Administration of the Slovak Republic.

Isolation of cells. Hepatocytes were isolated by the *in situ* two step collagenase perfusion technique as described by Michalopoulos et al. [10]. First, the liver was perfused with a Ca^{2+}-free buffer solution without collagenase (142 mM NaCl, 6.7 mM KCl in 10 mM HEPES buffer, pH 7.4). In the second step, the buffer solution was supplemented with Ca^{2+} (0.5 mM) and collagenase (0.5 mg/ml). Perfusion was performed through the *inferior vena cava*. The dispersed cells were then filtered through a nylon mesh (pore size 120 μm), washed twice with Ca^{2+}-free buffer solution, and centrifuged (500 rpm, 10 min.). Finally, the cells were suspended in Minimum Essential Medium (MEM, Sigma Chemical Company, St. Louis, MO) containing 1.8 mM Ca^{2+}. The cell viability assessed by trypan blue exclusion method was ~ 75-80%.

Testicular cells were isolated from rat testes of sexually mature male SD rats by enzymatic digestion as described by Bradley and Dysart [11], with some modifications suggested by Søderlund et al. [12]. Briefly, the testes were decapsulated and incubated at 32°C in RPMI medium (without fetal bovine serum, FBS) with collagenase (100 U/ml) for 20 min. Trypsin (2100 U/ml) was then added, and the tubular suspension was further incubated for 8 min. The resulting cell suspension was filtered, washed and resuspended in RPMI medium with FBS, centrifuged four times (1200 rpm, 5 min) and filtered through a nylon mesh (100 μm). Viability of isolated testicular cells measured by trypan blue exclusion was greater than 95%.

Single cell gel electrophoresis. The procedure of Singh et al. [13] was used with minor modifications suggested by Slameňová et al. [14, 15]. Briefly, 2 x 10^4 cells were spread on a base layer of 1% normal-melting point agarose placed on microscopic slide and covered with a cover slip. After solidification of the gel, the cover slip was removed. Treatment with hydrogen peroxide (0, 100, 150, 200, and 250 μM of H_2O_2 for 5 min on ice in the dark) was carried out with cells embedded in agarose on microscopic slides. The slides were then placed in lysis solution for 1 h at 4°C to remove cellular proteins. After lysis the slides were transferred to an electrophoresis buffer (pH > 13) for 40 min unwinding time at 4°C and then subjected to electrophoresis at 25 V (current adjusted to 300 mA) for 30 min at 4°C. After electrophoresis the slides were neutralized with Tris-HCl two times for 10 min and stained with ethidium bromide (EtBr, Sigma Chemical Company, St. Louis, MO, 5 μg/ml). EtBr stained nucleoids were evaluated with an Olympus BX-51 fluorescence microscope

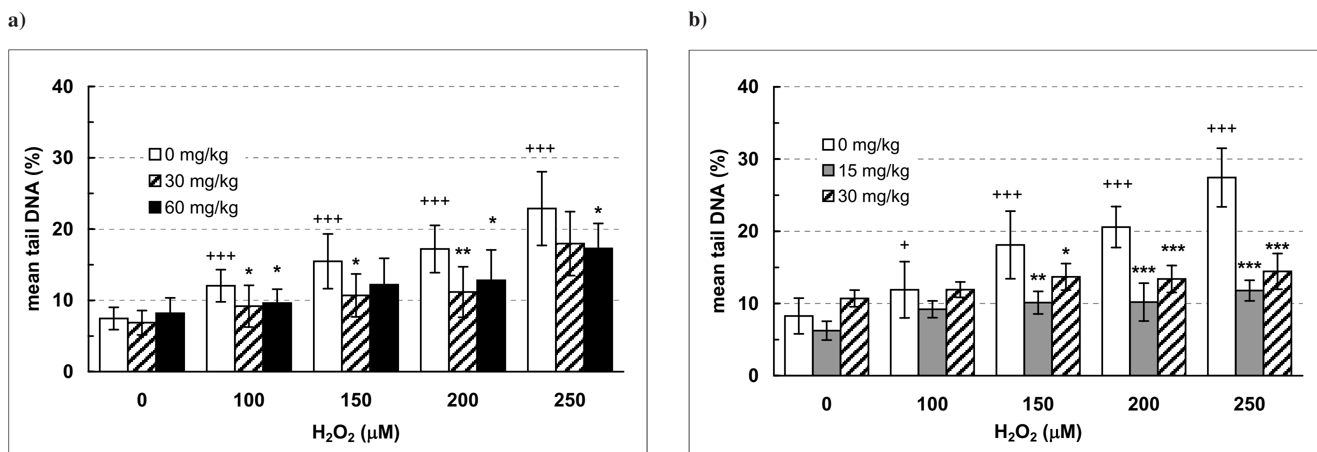

Fig.1a and Fig.1b. These figures represent the level of DNA lesions induced in rat hepatocytes freshly isolated from control rats (open bars; Fig. 1a and Fig. 1b); rats supplemented with 30 and 60 mg/day/kg b.w of carvacrol for 7 days (Fig. 1a, striped and black bars); and rats supplemented with 15 and 30 mg/day/kg b.w. of carvacrol for 14 days (Fig. 1b, grey and striped bars). Level of DNA lesions (strand breaks) was measured by single cell gel electrophoresis. Results are mean of two independent isolations ± SD. $^{+}$ p < 0.05, $^{+++}$ p < 0.001 refers to difference between untreated hepatocytes and hepatocytes treated with different concentrations of H_2O_2; * p < 0.05, ** p< 0.01, *** p < 0.001, refer to difference between values measured in H_2O_2 – treated hepatocytes isolated from control rats and H_2O_2 – treated hepatocytes isolated from carvacrol supplemented rats.
Statistical analysis by general linear model for repeated measures showed a statistically significant:

1. **DNA-damaging effect of all H_2O_2 concentrations in hepatocytes isolated from control rats (0 mg/day/kg b.w.); Increase of DNA lesions (between each of two H_2O_2 concentrations) was not significant only between concentrations 150 and 200 μM (Fig. 1a and Fig. 1b).**
2. **difference in H_2O_2-induced DNA damage among groups of hepatocytes with different dose of carvacrol. Control hepatocytes had significantly higher values of DNA damage than hepatocytes isolated from carvacrol-supplemented animals (Fig. 1a and Fig. 1b).**
3. **influence of interaction between concentration of H_2O_2 and dose of carvacrol; Increase of H_2O_2 from 100 μM to 150 μM and from 200 μM to 250 μM led to a statistically significant increase of DNA lesions in controls as regards to carvacrol groups. There was also a statistically significant difference in DNA damage between groups with different carvacrol doses. The highest values were found in controls and the lowest in the samples coming from animals supplemented by 15 mg/day/kg b.w. (Fig. 1a and 1b).**

(Olympus Europa, Hamburg, Germany). For each sample, 100 comets were scored by computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of DNA in the tail, linearly related to the frequency of DNA strand breaks.

Statistics. The results represent a mean from 2 sets of experiments N°1 and N°2. The significance of differences between samples was evaluated by Student's *t*-test (statistically decreased from untreated animals – "common diet"- $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). For modeling of relationship between DNA damage (dependent variable) and all doses of carvacrol and concentrations of hydrogen peroxide (independent variables) we used general linear model for repeated measures.

Results

Induction of ss DNA breaks. As evident from Figs 1a and 1b (0 mg/day/kg b.w.) and 2a and 2b (0 mg/day/kg b.w.), the percentage of DNA in the tail, which is linearly related to the frequency of DNA strand breaks, equals in hepatocytes isolated from control rats < 10%, while in testicular cells < 20%. This means that the level of spontaneous DNA lesions is significantly higher in testicular cells than in hepatocytes. It has been shown that the activity of the enzyme catalase, which is responsible for effective protection of cells and cellular DNA against an attack of oxidants, is widely distributed in mammalian cells but it is highest in the liver [16]. Treatment of the freshly isolated control hepatocytes and testicular cells with H_2O_2 led to a dose-dependent increase of DNA breaks in both hepatocytes (Figs 1a and 1b, open bars) and testicular cells (Figs 2a and 2b, open bars). The percentage of tail DNA was at each comparable H_2O_2 concentration tested (100, 150, 200 μM) more than two times higher in testicular cells than in hepatocytes.

DNA–protective effects of carvacrol added to drinking water (ex vivo experiments). The aim was to determine whether the cells freshly isolated from experimental rats supplemented by carvacrol, would manifest higher resistance against hydrogen peroxide (H_2O_2; 100, 150, 200, and 250 μM) than cells isolated from control animals. The results presented in Figs 1a and 1b (hepatocytes) and 2a and 2b (testicular cells) showed that the level of H_2O_2-induced DNA strand breaks was significantly reduced in both hepatocytes and testicular cells isolated from rats supplemented by carvacrol for 7 days (30 and 60 mg/day/kg b.w.) or 14 days (15 and 30 mg/day/kg b.w.).

Discussion

Due to the environment humans live in, they are constantly confronted with genotoxic or potentially genotoxic com-

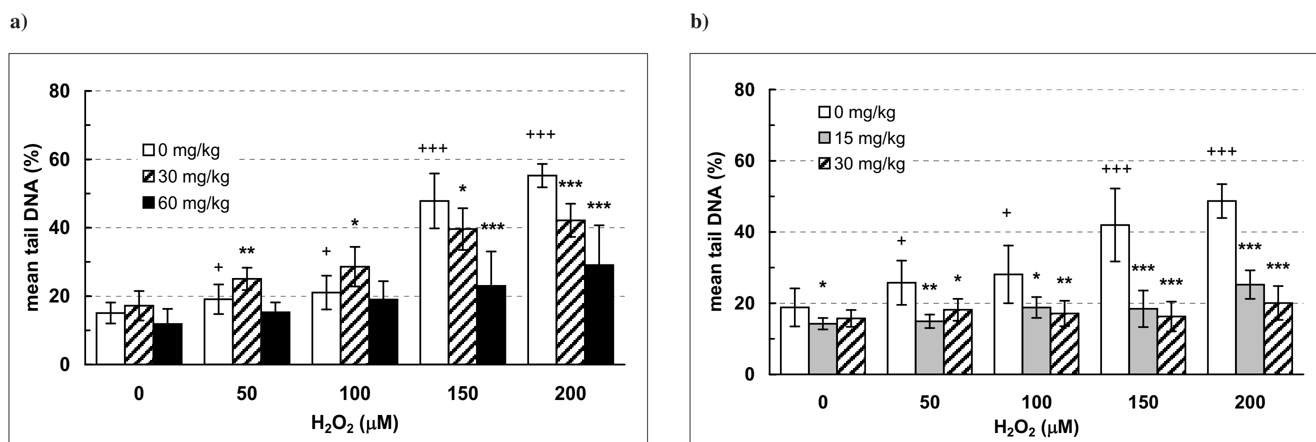

Fig.2a and Fig.2b. These figures represent the level of DNA lesions induced in rat testicular cells freshly isolated from control rats (open bars; Fig. 2a and Fig. 2b); rats supplemented with 30 and 60 mg/day/kg b.w. of carvacrol for 7 days (Fig. 2a, striped and black bars); and rats supplemented with 15 and 30 mg/day/kg b.w. of carvacrol for 14 days (Fig. 2b, grey and striped bars). Level of DNA lesions (strand breaks) was measured by single cell gel electrophoresis. Results are mean of two independent isolations ± SD. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ refer to difference between untreated testicular cells and testicular cells treated with different concentrations of H_2O_2 ; * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$, refer to difference between values measured in H_2O_2 – treated testicular cells isolated from control rats and H_2O_2 – treated testicular cells isolated from carvacrol supplemented rats. Statistical analysis of results by general linear model for repeated measures showed a statistically significant:

1. **DNA-damaging effect of all H_2O_2 concentrations in testicular cells isolated from control rats (0 mg/day/kg b.w.); Increase of DNA lesions (between each of two H_2O_2 concentrations) was not significant only between concentrations 50 and 100 μM in the fourteen-day-long experiment (Fig. 2b).**
2. **difference in H_2O_2-induced DNA damage among groups of testicular cells with different doses of carvacrol. Control testicular cells had significantly higher values of DNA damage than testicular cells isolated from carvacrol-supplemented animals (Figs 2a and 2b).**
3. **influence of interaction between concentration of H_2O_2 and dose of carvacrol; The increase of H_2O_2 concentration from 100 μM to 150 μM (Fig. 2a) and from 0 μM to 50 μM and 100 μM to 150 μM (Fig. 2b) led to a statistically significant increase of DNA damage in control as regards to two carvacrol groups. There was also a statistically significant difference in DNA damage between groups with different carvacrol doses. The highest values were found in control samples.**

pounds. These can cause DNA damage and gene mutations to human's somatic cells, which could sometimes give rise to cancer. When germinal cells are affected, the DNA damage could also have an effect on the next and successive generations. Freshly isolated hepatocytes (a representative of somatic cells) and testicular cells (a representative of germinal cells) have many advantages, which entitle their use for the study of mutagenic/antimutagenic and DNA-damaging/protective effects of different chemical compounds. As the liver is the main organ for the metabolism of foreign compounds, hepatocytes represent a suitable system for the evaluation of different effects which could potentially induce or reduce the response to mutagen. The examination of sperm or testicular cells seems to be an easier and logical choice for genotoxicity studies on germinal cells, as female germ cells are more complicated to obtain. The testicles comprise seminiferous tubules (hundreds of tiny tubes), Leydig cells (which is where testosterone is produced), and Sertoli cells (which are responsible for nurturing immature sperm cells. The term testicular cells includes therefore different types of cells. Primary cells as hepatocytes and testicular cells which are obtained directly from an animal usually maintain the differentiated state for a short period (hours to days).

Nature offers a wide range of DNA-protective natural compounds that can reduce or eliminate genetic damage induced by genotoxic agents. Numerous research studies testify that some plant compounds exert this role, but the mechanism that cause this effect has not been sufficiently explored. The use of plant extracts, e.g. essential oils (EOs), has a long tradition in human society, as these compounds manifest different remarkable biological effects (antibacterial, antiviral, insecticidal, antiinflammatory, antioxidative) and have preventive and therapeutic effects against many diseases [17, 18]. As early as in 1994, Aeschbach et al. [19] observed that thymol, carvacrol, 6-gingerol and hydroxytyrosol were good scavengers of peroxyl radicals and decreased peroxidation of phospholipid liposomes in the presence of iron(III) and ascorbate. The authors suggested to use the antioxidant properties of the compounds studied as a natural replacement for synthetic antioxidant food additives. Sokmen et al. [20] found that antimicrobial and antifungal activities of the EO and various extracts from herbal parts and callus cultures of *Origanum acutidens* against bacteria, fungi and a yeast were accompanied by a moderate antioxidative capacity, proved by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability and beta-carotene/linoleic acid assays. In addition to *in vitro* studies, also some *in vivo* animal bioassays described the antioxidant status of plant extracts and their positive effects on animals. Lima et al. [21] proved (by quantification of plasma transaminase activity and liver glutathione-S-trans-

ferase – GST and glutathione reductase – GR activities) the antioxidant potential of a traditional water infusion (tea) of common sage (*Salvia officinalis L.*). The replacement of water by sage tea for 14 days in the diet of rodents, which did not affect either body weight, food consumption, or liver toxicity, increased significantly liver GST activity in rats and mice.

In our laboratory we demonstrated, that in the presence of a wide scale of the monotherpenes thymol and carvacrol, incubation of human hepatoma cells HepG2, colonic cells Caco-2 and lymphomatic cells K562 did not cause at $IC\leq_{20-50}$ any genotoxic effects, but led to a significant protection of cellular DNA against a strong oxidative agent, hydrogen peroxide [22, 23, 15]. Based on the obtained results, it is however not possible to determine whether these DNA-protective effects are operative also in the intact organism of experimental rats. The main aim of this work was therefore to determine the protective activity of carvacrol added in *in vivo* conditions against DNA lesions induced by the model oxidant hydrogen peroxide in freshly isolated rat primary hepatocytes and testicular cells. Results presented in this study showed that replacement of drinking water by carvacrol supplemented water for 7 days (30 and 60 mg/day/kg b.w.) or 14 days (15 and 30 mg/day/kg b.w.) led to a significant reduction of the level of DNA strand breaks induced by H_2O_2 in both hepatocytes and testicular cells. To date the exact mechanism in which carvacrol reduces genotoxic effects of H_2O_2 is not known and it is not possible to say whether these effects are based exclusively on antioxidative actions of the plant components tested, or are rather associated with stimulation of cellular DNA repair. The level of DNA base excision repair in hepatocytes and testicular cells isolated from control and supplemented rats following treatment with genotoxic agents will be investigated in our further studies. There is however substantial metabolic and experimental evidence to indicate that carvacrol belongs to the micronutrients which can decrease the risk of cancer development.

The use of mammalian cells cultured *in vitro* or *ex vivo* is very useful in investigating the ability of natural compounds to reduce or eliminate genotoxic effects of oxidative carcinogens. These systems have at their disposal a wide spectrum of protective mechanisms that shield important cellular biomacromolecules against toxic effects of oxidants, and thus cells cultured *in vitro* or *ex vivo* approach conditions in which they occur in living organisms.

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