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ANTIGENOTOXIC EFFECT OF LIGNIN PREPARATIONS FROM WASTES OF PULP AND PAPER INDUSTRY - *IN VITRO* AND *EX VIVO* ACTIVITY

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ABSTRACT

Lignin belongs to the main components of plant biomass. It is obtained as a by-product in the paper production and biomass-conversion technologies. Potential use of lignin preparations as antioxidants results from the presence of unique hindered phenolic hydroxyl groups which can act as radical scavenger. The cytotoxicity of lignin preparations isolated from spent liquors was examined by Plating efficiency as well as by Trypan blue exclusion technique on mammalian cells (hamster V79, human CaCo-2 and LEP cells). The cytotoxicity of lignins vary with wood species, methods of isolation and fractionation. Their genotoxicity was determined by comet assay. The *in vitro* activity lignin preparations was tested in mice lymphocytes and testicular cells against DNA damage induced by hydrogen peroxide. In the cells preincubated with lignin a significant decrease of DNA lesions induced by H_2O_2 can be observed in comparison with control sample. The ability of the lignin antioxidants to protect organisms against the development of cancer was examined on lignin fed rats in *ex vivo* experiments. The obtained results show that the used lignin sample decreased the level of strand breaks significantly in rat lymphocytes and testicular cells, i.e. their resistance to oxidative stress induced by H_2O_2 was increased. The revealed antigenotoxic effect of the isolated lignins *in vitro* and *ex vivo* shows new way of valorization and utilization of lignin waste products from pulp and paper industry for antitumor use. The results obtained by determination of cytotoxicity of lignins and by the experiments with laboratory animals indicate that non-toxic lignin preparations have potential to protect living organisms against damaging effects of different genotoxicants instead of synthetic antioxidants.

Keywords: lignin, cytotoxicity, DNA, *in vitro*, *ex vivo.*

INTRODUCTION

Lignin is amorphous cross-linked polymer composed of phenylpropane units. Its biological importance is very significant as it reinforces the plant against the forces of gravity and wind; it seals the water-conducting system and provides the plant with a protective barrier against microorganism attack. Lignin plays an important role in the natural ecology cycle: once in the soil, it serves as a complexing agent for minerals and as a moisture-retention aid (HIGUCHI 1980). Hindered phenolic hydroxyl groups of lignin can act as stabilizers of reactions induced by oxygen and its radical species (KRATZL *et al*. 1967). It has been show that lignins possess multiple properties such as antioxidant and antimicrobial activity (BARCLAY, XO, NORRIS 1997, LORA, GLASSER 2002, DIZHBITE *et al*. 2004). In our previous works (KOŠÍKOVÁ *et al.* KAČURÁKOVÁ 1993, KOŠÍKOVÁ, GREGOROVÁ 2005) the influence of lignin on rheological and strength properties of polyolefin composites and rubber vulcanizates was examined. The potential protective role of lignin biomass component against carcinogenesis was investigated by characterization of a binding affinity of lignin preparations towards different carcinogens (KOŠÍKOVÁ *et al.* 1990). In view of several drawbacks of synthetic compounds for the human

organism, the ability of lignin preparations isolated from waste products of wood pulping to protect DNA against oxidative damage was examined in our research mammalian cells and laboratory animals. The protective activity of lignin was tested in vitro, where lignin was added to isolated hepatocytes before treatment with H_2O_2, and *ex vivo*, where lignin was used as part of diet for experimental rats which served as a source hepatocytes treated after isolation with H_2O_2.

EXPERIMENTAL

A series of lignin preparations I-VI with: 15.2; 15.8; 17.1; 16.3; 15.9; 16.1 % OCH_3 and 6.5; 5.8; 6.4; 6.9; 5.7; 6.3 % phenolic OH was obtained by fractionation of spent liquors derived from kraft pulping of softwood. Sulfur free lignin preparation with 19.1 % OCH_3 and 4.3 % phenolic OH was obtained by fractionation of by-products of hardwood prehydrolyzate.

Cell lines: Quasidiploid Chinese hamster V79 cells, lung fibroblasts as well as diploid human LEP cells, lung embryonic fibroblast obtained from The National Institute of Public Health, Prague, Czech Republic were cultured in RPMI 1640 medium (with 25mM HEPES and L-glutamine) supplemented with 10 % fetal calf serum and antibiotics penicillin (100 U/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml). Cells were incubated in plastic Ru-flasks or plastic 4-well and 12-well plates in a CO_2 incubator at 37 °C.

Plating efficiency test: One hundred V79 cells were plated in one well of 4-well plate (diameter = 3cm) and incubated for 3−4 h. The medium was removed and cells were 2 hour treated with lignin samples (25, 50, 100, 200 and 400 μg/ml). Control cells were kept in a fresh RPMI medium. After treatment, the cells were washed with PBS and then cultivated for 7 days in fresh RPMI medium to estimate cloning efficiency. The percent of plating efficiency of cells was calculated after staining of colonies with methylene blue (1% solution in distilled water).

Trypan blue exclusion technique: The V79 cells as well as LEP cells growing on wells of 12-well plate in monolayers were exposed to different concentration of lignin samples (25, 50, 100, 200 and 400 μg/ml). Control cells were kept in fresh RPMI medium. The number of cells in a control group as well as in treated groups was counted after trypan blue staining (0.4 %). The number of viable cells (%) was determined.

Testicular cells from mice were isolated from mouse testes of sexually mature male Balb/c mice by enzymatic digestion, as described by BRADLEY and DYSART (1985), with some modifications (LÅG *et al*. 1989). The total yield per mouse testes was about $1.5{-}2 \times 10^7$ cells with viability greater than 95 %, as measured by trypan blue exclusion. Blood lymphocytes were isolated from fresh heparinized blood taken directly from the heart of male mice by standard gradient technique at a density of 1.077 g/ml. The viability of lymphocytes measured by trypan blue exclusion was > 95 %. Testicular cells and lymphocytes isolated from mice were incubated for 2 h with lignin (50 μg/ml) while being continuously shaken at 32 °C (testicular cells) or 37 °C (lymphocytes) in complete RPMI medium. The level of DNA strand breaks was measured using single-cell electrophoresis, i.e., comet assay. The comet assay is based on the ability of DNA strand breaks for strands to migrate in a weak electric field in the direction of the anode, giving the nucleolus the appearance of the tail of a comet when visualized by fluorescence microscopy.

In the experiments with cells isolated from Balb/c mice, lignin was dissolved in 100 % DMSO (10mg/ml) and diluted in complete RPMI 1640 medium (final lignin concentration – 50 μg/ml) shortly before use. Hydrogen peroxide, H_2O_2 (Chemické závody Sokolov, Czech Republic), was diluted in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free) to final concentrations of 50, 75, 100, 200, and 400 μmol/l 1 minute before use and kept at 4 ºC. This stock solution was diluted shortly before use in PBS buffer to the final concentration of 3.125×10^{-5}mol/l and kept at 4 ºC.

The male Sprague-Dowley rats obtained from ANLAB, Czech Republic were housed two per cage under standard environmental conditions (22 ± 2°C, 55 ± 5 % relative humidity, and

lights on from 06:00 to 18:00) insolid plastic cages on hardwood bedding. The rats were fed daily by a standard diet or a lignin-supplement diet (10g/100g body wt/day) for 21 days. All experiments were carried out in triplicate. Rat primary hepatocytes were isolated from rats using an *in situ* two-step collagenase perfusion technique as described by MICHALOPOULOS *et al*. (1982).

RESULTS AND DISCUSSION

The use of lignin has been receiving increase attention because of some advantages, such as its antioxidative nature and relative availability as by-product in the paper industry approximately 50×10^6 t of technical lignins per year (GLASSER 1981). Therefore in our experiments, a series of lignin preparations derived from softwood kraft pulping was investigated for possible application as natural polymeric antioxidants. At the first these lignins were examined with the respect to their cytotoxicity on hamster cells V79 and human LEP cells by Plating efficiency test and Trypan blue exclusion technique for prospective application as natural antioxidants. The obtained results are summarized in Figures 1–3.

Figures 1−3 represent the results from experiments in which we tried to find some non-toxic or mildly toxic concentration of lignin samples. Influence of 2 h incubation of cells with lignin (0, 25, 50, 100, 200 and 400 μg/ml) was assayed by the Plating efficiency test in hamster V79 cells and by the Trypan blue exclusion technique in hamster V79 cells as well as in human LEP cells. Although it is assumed that in the plating efficiency test, one visible colony outgrows from each surviving cell, the Trypan blue exclusion technique is an indicator of cell death, membrane lysis, and significant uptake of vital dye (Trypan blue). After 2-h incubation with different concentration of lignin, V79 cells as well as LEP cells manifest a dose-dependent decrease in number of colonies and number of viable cells at concentration higher than 50 μg/ml.

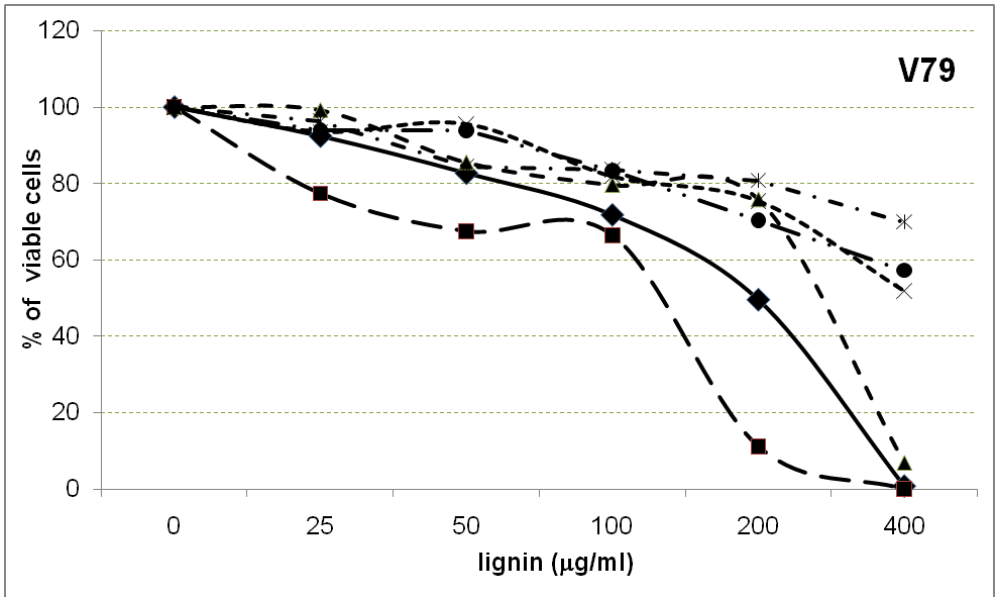

Fig. 1 Percentage of viable hamster V79 cells after treatment with lignin evaluated by the Plating efficiency test. Rhomb – Lignin I, square – Lignin II, triangle – Lignin III, cross – Lignin IV, star – Lignin V and circle – Lignin VI.

Obr. 1 Percento vyrastených kolónií škrečacích V79 buniek po spracovaní s lignínom stanovené PE (Plating efficiency) testom. Kosoštvorec – Lignín I, štvorec – Lignín II, trojuholník – Lignín III, krížik – Lignín IV, hviezdička – Lignín V a kruh – Lignín VI.

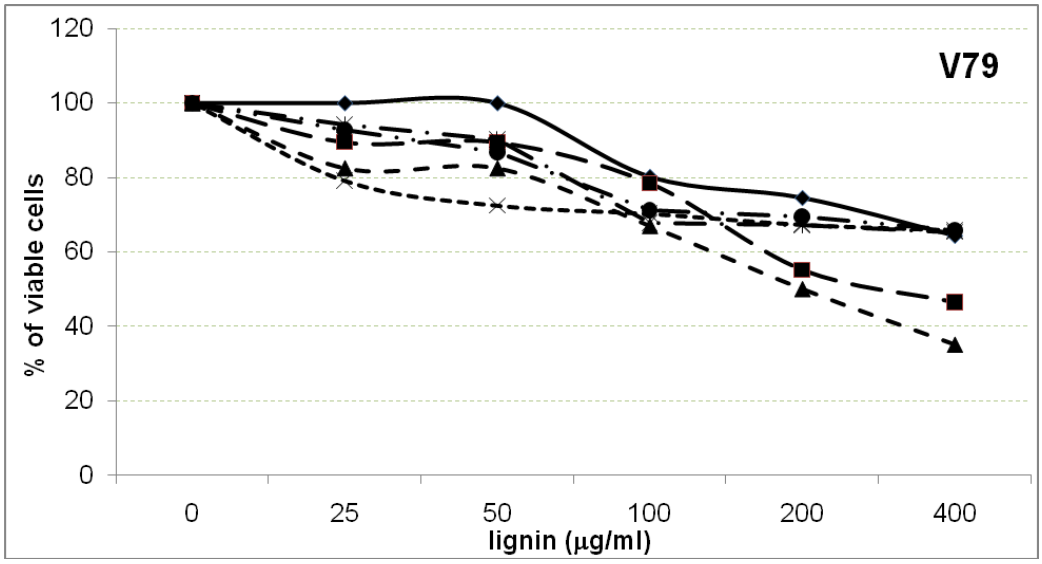

Fig. 2 Percentage of viable hamster V79 cells after treatment with lignin evaluated by the Trypan blue exclusion technique. Rhomb – Lignin I, square – Lignin II, triangle – Lignin III, cross – Lignin IV, star – Lignin V and circle – Lignin VI.
Obr. 2 Percento živých škrečacích V79 buniek po spracovaní s lignínom stanovené TBET (Trypan blue exclusion technique) testom. Kosoštvorec – Lignín I, štvorec – Lignín II, trojuholník – Lignín III, krížik – Lignín IV, hviezdička – Lignín V a kruh – Lignín VI.

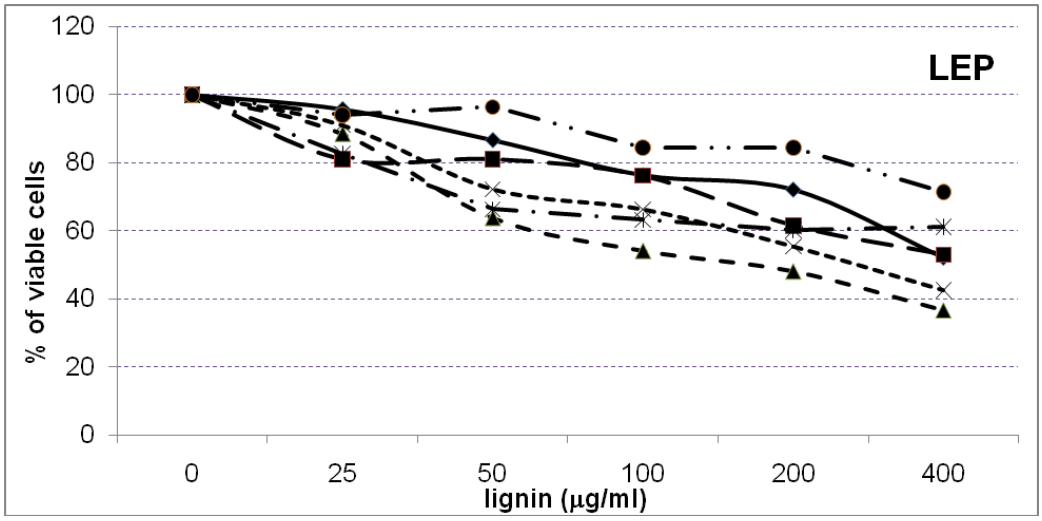

Fig. 3 Percentage of viable human LEP cells after treatment with lignin evaluated by the Trypan blue exclusion technique. Rhomb – Lignin I, square – Lignin II, triangle – Lignin III, cross – Lignin IV, star – Lignin V and circle – Lignin VI.
Obr. 3 Percento živých ľudských embrionálnych pľúcnych buniek po spracovaní s lignínom stanovené TBET (Trypan blue exclusion technique) testom. Kosoštvorec – Lignín I, štvorec – Lignín II, trojuholník – Lignín III, krížik – Lignín IV, hviezdička – Lignín V a kruh – Lignín VI.

Using the Plating efficiency test in V79 cells it was observed, that the most toxic lignin samples are lignins I, II and at the highest concentration the lignin III as well in comparison with lignin IV−VI. Using the Trypan blue exclusion technique it was observed similar outcomes in

both cell lines. Less than 80 % of viable V79 cells as well as LEP cells were observed at concentration higher than 100 μg/ml of all lignin samples using both, the Plating efficiency test and the Trypan blue exclusion technique. The non-toxic lignin preparations IV–VI have potential for application as natural human diet additives.

The ability of lignin preparation derived from beech wood prehydrolysis to protect organisms against the development of cancer was examined on the cells isolated from mice in *in vitro* experiments. Figure 4 represents the results of lignin characterization with the respect to its cytotoxicity on human carcinoma CaCo-2 cells. Influence of 2 h incubation with various concentration of lignin was assayed by Trypan blue exclusion technique. The obtained results show that CaCo-2 cells decreased in the number of viable cells at concentrations higher than 50 μg/ml. Therefore 2h preincubation of all isolated cells with 50 μg/ml was used in further experiments. The determination of genotoxicity of lignin tested by the comet assay confirmed that incubation of cells with lignin has no genotoxic effects.

The protective effect of this lignin preparation towards damage of deoxyribonucleic acid (DNA) *in vitro* generated by hydrogen peroxide on mice lymphocytes and testicular cells was examined. Figure 5 presents the level of direct DNA strand breaks in H_2O_2-treated testicular cells and the level of H_2O_2-induced DNA strand breaks in cells preincubated for 2h with 50 μg/ml of lignin. In cells preincubated with lignin a significant decrease of DNA lesions induced by H_2O_2 can be observed in comparison with control sample. Figure 6 shows that lymphocytes were more sensitive to the effect of hydrogen peroxide than testicular cells. The obtained results confirm that lignin-pretreatment decreased the level of DNA strand breaks in mice testicular cells and lymphocytes, i.e., the resistance of testicular cells and lymphocytes to oxidative stress induced by hydrogen peroxide was increased.

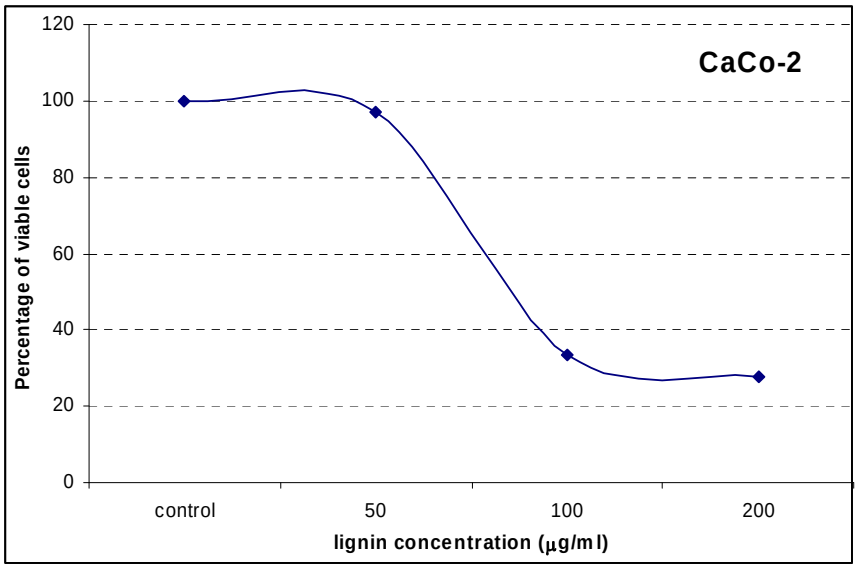

Fig. 4 Percentage of viable cells after treatment with lignin evaluated by the Trypan blue exclusion technique in human CaCo-2 cells. Values represent the mean of 3 independent experiments. Statistical in significance as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.
Obr. 4 Percento živých ľudských karcinogénnych buniek CaCo-2 po spracovaní s lignínom stanovené TBET (Trypan blue exclusion technique) testom. Uvedené hodnoty reprezentujú priemer 3 nezávislých experimentov. Štatistická významnosť je nasledovná: *ρ < 0.05, **ρ < 0.01, and * ρ < 0.001.**

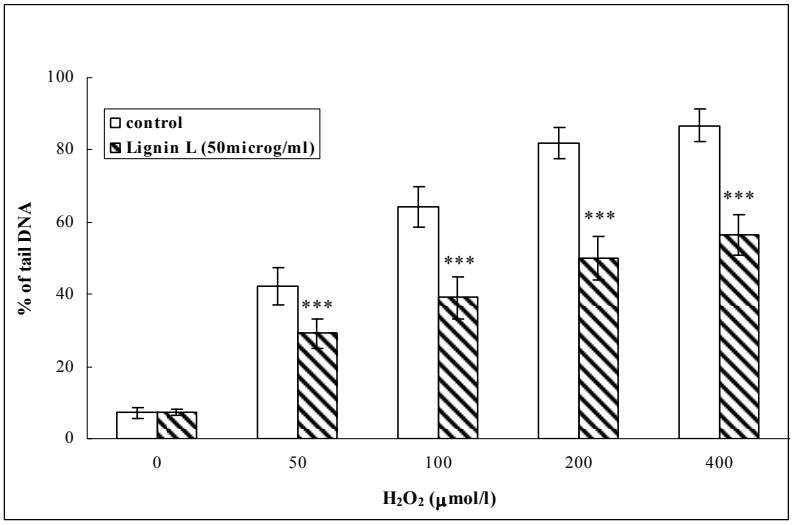

Fig. 5 Decrease in H_2O_2-induced DNA damage in testicular cells from mice by lignin. Values represent the mean of 3 independent experiments. Statistical in significance as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

Obr. 5 Redukcia oxidačného poškodenia DNA v testikulárnych bunkách izolovaných z myší účinkom lignínu. Uvedené hodnoty reprezentujú priemer 3 nezávislých experimentov. Štatistická významnosť je nasledovná: *ρ < 0.05, **ρ < 0.01, and * ρ < 0.001.**

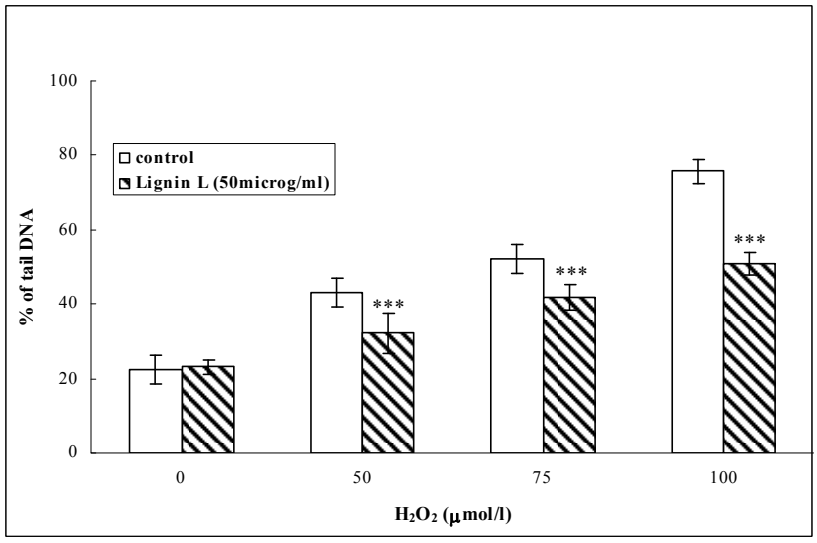

Fig. 6 Decrease in H_2O_2-induced DNA damage in lymphocytes from mice by lignin. Values represent the mean of 3 independent experiments. Statistical in significance as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

Obr. 6 Redukcia oxidačného poškodenia DNA v lymfocytoch izolovaných z myší účinkom lignínu. Uvedené hodnoty reprezentujú priemer 3 nezávislých experimentov. Štatistická významnosť je nasledovná: *ρ < 0.05, **ρ < 0.01, and * ρ < 0.001.**

Protective activity of lignin against DNA lesions induced in rats fed by diet containing lignin was examined *in vitro* and *ex vivo.* Freshly isolated rat hepatocytes are an excellent model for the screening of genotoxicants as well as the investigation of protective effects of different

antioxidants. They maintain complete metabolic competence for bioactivation and degradation of xenobiotics. In *in vitro* experiments, freshly isolated hepatocytes were incubated with lignin for 2 h and after incubation the cells were subsequently treated with H_2O_2. In *ex vivo* experiments hepatocytes were isolated from rats fed a standard diet (without lignin) and rats fed a diet containing 8 wt% lignin.

Figure 7 shows that the level of single strand DNA breaks increased proportionally with increasing concentration of H_2O_2 in all types of hepatocytes studied; however, the level of DNA breaks was significantly lower in hepatocytes pre-incubated with lignin either *in vitro* or *ex vivo*. In *ex vivo* experiments, the cells were exposed to the same model genotoxin after isolation from rats fed lignin supplemented diet. Figure 7 represents the lignin-stimulated reduction of direct DNA strand breaks induced by hydrogen peroxide (0, 200, and 400 μmol/l) in primary hepatocytes. As it is evident, lignin caused a significant decrease of hydrogen peroxide-induced DNA strand breaks both in *in vitro* conditions and in *ex vivo* conditions, i.e., when given to Sprague-Dawley rats in diet.

The mechanisms of the protective effect of lignin have not been elucidated (clearly antioxidant effects do not explain everything). Based on recent literature data (BEGUM 2004) lignin is partially digested in gut microflora in lignans, which can diffuse into cells and so decrease oxidative damage DNA.

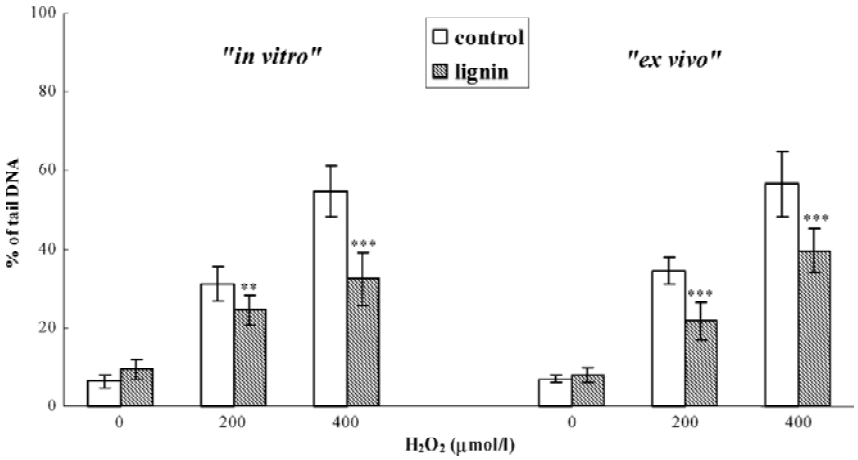

Fig. 7 Influence of preincubation of rat hepatocytes with lignin on the level of DNA strand breaks induced by hydrogen peroxide *in vitro*. Effect of dietary intake of 8% lignin on the level of DNA strand breaks induced in rat hepatocytes by hydrogen peroxide *ex vivo*. Data represent the mean of 3 independent experiments (with 5 parallels each) ± standard deviation. Statistical significance (by *t* - test): **p < 0.01, ***p < 0.001.

Obr.7 Vplyv predspracovania hepatocytov izolovaných z potkanov s lignínom na redukciu poškodenia DNA indukovaného peroxidom vodíka *in vitro*. Vplyv prídavku lignínu do potravy pre potkany na redukciu poškodenia DNA indukovaného peroxidom vodíka *ex vivo*.

CONCLUSION

The observed reduction of H_2O_2-induced DNA lesion in animals consuming a lignin-containing diet is very promising for medical applications. The revealed reduction of DNA breaks by lignin indicates that it belongs to the micronutrients which can decrease the risk of cancer development. The results obtained by the Plating efficiency test and Trypan blue exclusion technique showed that non-toxic kraft lignin preparations isolated from wastes of pulp

and paper industry have potential to protect living organisms against damaging effects of different genotoxicants instead of synthetic antioxidants.

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