LIGNIN-STIMULATED PROTECTION OF POLYPROPYLENE FILMS AND DNA IN CELLS OF MICE AGAINST OXIDATION DAMAGE

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The blending of polypropylene with lignin derived from chemical wood pulp manufacture makes it possible to prepare optically transparent films (thickness 50-60μm) with acceptable mechanical properties in the absence of a commercial stabilizer. The lignin preparation in the concentration 1-2 wt% possessed the ability to act as a processing stabilizer and as an antioxidant during thermal aging of polypropylene films. A DNA-protective effect of lignin in mice testicular cells and mice peripheral blood lymphocytes against oxidation stress was examined using *in vitro* experiments. Hydrogen peroxide and visible light-excited methylene blue (MB) were used as DNA damaging agents. The isolated cells were preincubated with lignin before treatment with the oxidative agents. The level of breaks in the DNA was measured by a comet assay. The results showed that preincubation with lignin significantly decreased the level of strand breaks induced by both oxidants in mice lymphocytes and testicular cells.

Keywords: Lignin antioxidant; Polypropylene films; Thermo-oxidative aging; DNA

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INTRODUCTION

It is known that polymeric materials and living organisms are susceptible to oxidative degradation. There are many factors causing polymer degradation – solar light or other light energy radiation, heating, chemical attack, etc. Generally, this degradation is understood to involve free radical species. Therefore, major attention has been devoted just to free radical scavenging additives, especially phenolic and amine antioxidants (Gugumus 2000). Recently, some possible negative effects of some plastics have been described regarding human health and the environment. Such effects may be caused not by the polymers themselves, but the stabilizers incorporated within them. The essential requirements for antioxidants are good solubility, mobility, low volatility and stability. However, the antioxidants exert their stabilizing effect even in landfills after expiry of the service life. This is not desired, since most of the synthetic stabilizers decelerate the decomposition of the plastic waste (Akovali 2007). Nowadays, new criteria for chemicals are required, such as environmental friendliness, nontoxicity, economical accessibility, and favorable waste disposal characteristics.

Lignins are complex phenolic polymers that occur in higher plant tissues. They act as antioxidants against the chemical, biological, and mechanical stresses to which plants are subjected. Chemical treatment of wood for pulp production yields approximately 50 x 10^6 t of technical lignins per year (Glasser 1981). Hindered phenolic

hydroxyl groups of lignin can act as stabilizers of reactions induced by oxygen and its radical species (Kratzl et al. 1967). It was revealed that the antioxidant activity of lignins varies with their genetic origin, isolation methods, and molecular characteristics. Lignin's ability to scavenge the radicals responsible for the oxidation is influenced by limited diffusion into polymer matrix (Pouteau et al. 2003). It has been shown that lignins possess multiple properties such as antioxidant and antimicrobial activity (Barclay et al. 1997; Lu et al. 1998; Lora and Glasser 2002; Dizhbite et al. 2004; Raghuraman et al. 2005; Drankhan et al. 2003). The use of lignin has been receiving increased attention because of some advantages, such as its relative availability as by-product in the paper industry, and of some properties, such as its cross-linked structure with tetrafunctional branch points, strong intramolecular interactions, processing ability, and biodegradability. In our previous work (Košíková et al. 2001) the influence of lignin (10, 20, and 30 wt %) on rheological and strength properties of polyolefin composites was examined. It was revealed that with increasing lignin content the mechanical properties of these composites deteriorated. Thus, the objective of the present study was examination of the antioxidant efficiency of lignin preparations derived from chemical wood treatment in multiple processing and thermo-oxidative aging of polypropylene films in the amount up to 5 wt%. In view of several drawbacks of synthetic compounds for the human organism, the ability of lignin polymeric antioxidants to protect DNA against oxidative damage was examined in the testicular cells and lymphocytes isolated from mice *in vitro*.

EXPERIMENTAL

Water-soluble sulfur-free lignin preparation (L_1) of average molecular mass Mw 2000 and polydispersity 1.2 with 19.1% OCH_3 and 4.3% phenolic OH was obtained by fractionation of by-products of hardwood prehydrolysis (170ºC) with a dioxane/water mixture (9:1) followed by purification according to Björkman (1956). Carbohydrate portion of the isolated lignin (9.3%) consists of glucose and xylose. Kraft lignin (L_2) of average molecular mass 6800 with 15.2% OCH_3 and 6.5% phenolic OH was precipitated from the concentrated spruce spent liquor (total solid 59.9%, ash 27.2%) with diluted sulphuric acid (25ml sulphuric acid and 375ml water). Gel permeation chromatography was performed on a column (53 × 8 cm) of Sephadex LH 60 using a mixture of dioxane and water containing 0.005mol/l of aqueous NaOH and 0.001mol/l of LiCl (7:3) as the eluant (Košíková and Mlynár 1992). Phenolic hydroxyl groups were determined by FTIR spectroscopy (Faix et al. 1992). All analyses were performed in triplicate.

A commercial polypropylene (Moplen 500N, Slovnaft, Bratislava, Slovak Republic) was mixed and extruded with 0.5% of Irganox 1010 (pentaerythrityl terakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate]) or 1, 2, and 5 wt % of lignin, respect-tively, using a Brabender Plasticorder at 200ºC. Extrusion was repeated six times. Polypropylene without stabilizer was treated in the same way as a control. The MFI was evaluated at 200ºC using a Melt flow T.Q.CEAST 6841 instrument according to ASTM D 1238. The extrudates obtained were disintegrated for subsequent injection molding. Films about 50-60μm were molded from the mixture at 200ºC. Tensile strength and

elongation data were obtained with an Instron tester according to standard STN EN ISO 527-1.3. Thermal aging was performed at 100ºC in an air-circulating oven for 168 h.

The antioxidant activity of lignin samples was determined by the luminal-dependent photochemical method (Photochemiluminometer, Analytic Jena AG, Jena, Germany) with an ACW kit. The method is based on the luminal-dependent generation and detection of superoxide anion radicals. The formation of superoxide anion radicals in this assay is characterized by a lag_o phase of the chemiluminiscent curve (Popov and Lewin 1994). In the presence of antioxidant, the lag phase is shifted to the light and the difference lag - lag_o phase is dependent on the antioxidative ability of the compounds tested. Trolox, a derivative of α-tocopherol (vitamin E), was used in our assay as a standard. The value of lag - lag_o obtained for the compounds tested was compared with the antioxidative ability of Trolox, and the antioxidative activity was defined as mmol Trolox exhibiting the same ability to shift the lag phase as our tested compounds. From the linear dependence, expressed by the straight-line equation $y = ax + b$ (where x is the lignin concentration in mmol and y is mmol Trolox related to the same lag - lag_o value as our tested compounds), the amount of lignin that gives the same lag - lag_o value as 1mmol Trolox was calculated ($x=(1-b)/a$).

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 - 50μg/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 400μmol/l 1 minute before use and kept at 4ºC. Methylene Blue (MB, product of Loba Feinchemie, Austria) was dissolved in PBS buffer at room temperature at the concentration 3.125×10^{-2}mol/l. 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 4ºC. Formamidopyrimidine-DNA-glycosylase (FPG) and Endonuclease III (Endo III) were obtained from A. Collins (Rowett Research Institute, Aberdeen, Scotland). The crude extracts of FPG and Endo III were diluted in 40mmol/l Hepes-KOH, 0.1mol/l KCl, 0.5mmol/l EDTA, 0.2mg/ml bovine serum albumin, pH = 8.0 (1:3000 and 1:1000, respectively) just before use.

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). After isolation, cells were kept in RPMI 1640 medium (GIBCO^{TM}, UK) supplemented with fetal calf serum (FCS, 10%), antibiotics (100 U/ml penicillin; 100 μg/ml streptomycin), and pyruvate (0.1 mg/ml). In short, testes were decapsulated and incubated at 32°C in RPMI medium (without FCS) with collagenase (100U/ml) for 15 min. Trypsin (2,750 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 FCS for stopping trypsinization, centrifuged four times (1,200 g for 8min), and filtered through nylon mesh. The total yield per mouse testes was about 1.5-2 x 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. Control cells were cultured without lignin but with 0.5% DMSO (concentration of DMSO in lignin samples). Before treatment with H_2O_2 or visible light + MB, the medium was removed, and treated cells were rinsed with fresh PBS and tested for the level of DNA breaks using single cell gel electrophoresis. Testicular cells (4 x 10^4) and lymphocytes (3 x 10^4) were suspended in 0.75% LMP (low melting point) agarose and spread on a base layer (100μl of 0.75% NMP (normal melting point) agarose in Ca^{2+}- and Mg^{2+}- free PBS on a microscopic slide. Cells embedded in the agarose gels were treated either with 50μl of H_2O_2 solution (concentrations: 50, 75, 100, 200, and 400μmol/l; 5 minutes at 4°C) or with visible light (60W bulb; 120 or 180s; 25cm distance) + MB (3.125×10^{-5}mol/l) on ice without any other source of light. A similar light exposure regimen was used by Hartwig et al. (1990) to induce DNA damage subsequently assayed with alkaline elution. Control cells were treated with MB for 180s only. After treatment the cells were washed with PBS 2 times for 2 minutes.

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. The procedure of Singh et al. (1988) was used with minor changes suggested by Slameňová et al. (1997) and Gábelová et al. (1997). Testicular cells or lymphocytes embedded in agarose gels situated on slides were treated with H_2O_2 or visible light + MB. Immediately after treatment the slides were placed in lysis solution (2.5mol/l NaCl, 100mmol/l Na_2EDTA, 10mmol/l Tris, pH = 10 and 1% Triton X-100) for 1 hour at 4ºC to remove cellular proteins. In the experiments studying the nature and characteristics of visible light-induced oxidative DNA damage, we used the modified comet assay suggested by Collins et al. (1993). After lysis the slides were washed two times for 10 minutes in endonuclease buffer (40mmol/l HEPES-KOH, 0.1mol/l KCl, 0.5mmol/l Na_2EDTA, 0.2mg/ml BSA, pH = 8) and incubated for 30 minutes with a mixture of the enzymes FPG and Endo III at 37ºC. The slides were then transferred to an electrophoresis box containing an alkaline solution at pH > 13 (300mmol/l NaOH, 1mmol/l Na_2EDTA, pH > 13) and kept in this solution for 40 minutes at 4ºC for DNA strands to unwind. A voltage of 25V (current of 300mA) was applied for 30 minutes. The slides were removed, neutralized by 2 × 10 minute washing in Tris-HCl (0.4mol/l, pH = 7.5), and stained with 20μl ethidium bromide (EtBr, 10μg/ml). EtBr stained nucleoids were evaluated with a Zeiss Jenalumar fluorescence microscope. For each sample 100 comets were scored by computerized image analysis (Komet 5.5 Europe, Kineting Imaging, Liverpool, UK) for determination of DNA in the tail, linearly related to the frequency of DNA strand breaks (McKelwey-Martin et al. 1993).

Statistical Analysis

The significance of differences between samples (without lignin and with lignin) was evaluated by the Student's t-test, which in all assays used was statistically different from the corresponding control: *** $p < 0.001$.

RESULTS AND DISCUSSION

The antioxidant activity of tested lignins determined by the luminal-dependent photochemical method was 0.230±0.004mmol Trolox equivalent for L_1 and 0.311±0.004mmol Trolox equivalent for L_2, respectively. The processing stability of the prepared polypropylene blends was evaluated by measuring of the melt flow index (MFI) as a function of the extruded polymer blends for a given number of extrusions (Fig.1). Both Irganox and lignin sample strongly decreased oxidative degradation of the PP matrix. Lignin addition to Irganox enhanced its antioxidant efficiency probably due to ability of lignin to modify surface properties of PP described in our previous paper (Košíková et al. 1995). Based on this, an increased diffusion of Irganox into polymer matrix and its lower volatility could be suggested.

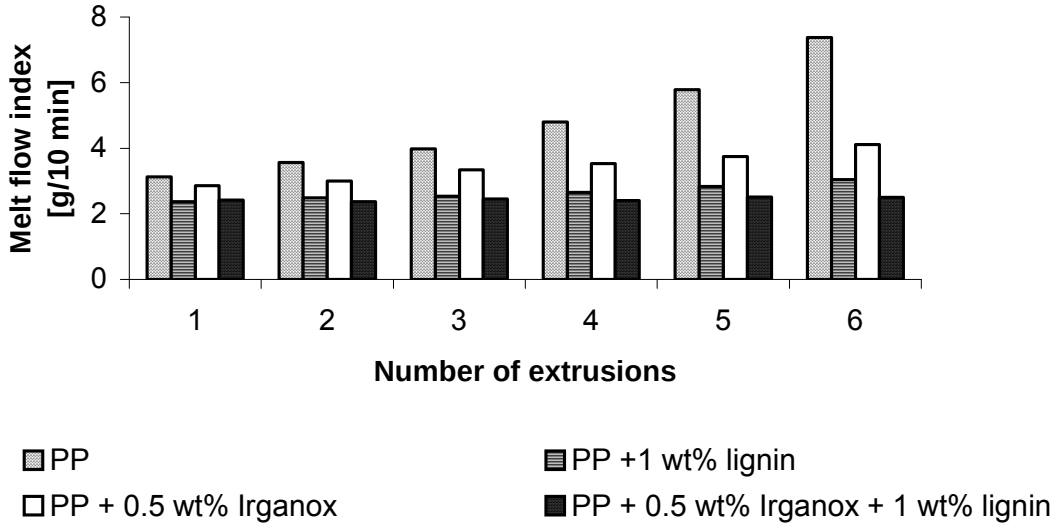

Fig. 1. Effect of number of extrusions on melt flow index of polypropylene blends. (Values represent the mean of at least six independent experiments.)

Further experiments were focused on the behaviour of PP films containing lignin L_1 and L_2, respectively, in the amount 0.5-5 wt % during thermo-oxidative aging. The mechanical properties (tensile strength at break and elongation at break) of the prepared films before and after aging are presented in Tables 1 and 2.

The summarized data show that PP films containing up to 2 wt % lignin were rather stable towards thermo-oxidation in comparison with lignin-free film containing the commercial stabilizer. The results obtained demonstrate that the lignins used can act as effective antioxidants during thermo-oxidative aging of polypropylene films. In contrast, the films containing 5 wt % lignin exhibited lower mechanical properties.

Table 1. Mechanical Properties of Polypropylene Films Containing Lignin (L_1) Before and After Oven Aging (Values represent the mean of at least five independent experiments.)

Sample composition	Tensile strength at break [MPa]		Elongation at break [%]	
	Before aging	After aging	Before aging	After aging
PP	13.5	15.7	589.4	335.3
PP + 0.5 % L_1	14.6	18.4	541.2	374.7
PP + 1 % L_1	13.3	20.1	541.5	569.1
PP + 2 % L_1	12.9	18.9	497.3	504.5
PP + 5 % L_1	6.0	6.2	109.6	37.5

Table 2. Mechanical Properties of Polypropylene Films Containing Lignin (L_2) Before and After Oven Aging (Values represent the mean of at least five independent experiments.)

Sample composition	Tensile strength at break [MPa]		Elongation at break [%]	
	Before aging	After aging	Before aging	After aging
PP	13.5	15.7	589.4	335.3
PP + 0.5 % L_2	12.8	19.0	556.2	485.5
PP + 1 % L_2	14.5	21.9	573.2	622.1
PP + 2 % L_2	13.6	20.6	494.0	597.7
PP + 5 % L_2	4.5	5.1	71.1	33.0

The ability of a lignin polymeric antioxidant to protect organisms against the development of cancer was examined on the cells isolated from mice in *in vitro* experiments. Figure 2 represents the results from experiments in which we tried to find some appropriate concentration of lignin sample. Influence of 2 h incubation of cells with lignin (50, 100, and 200 μg/ml) was assayed by Trypan blue exclusion technique in CaCo-2 cells. Trypan blue exclusion technique is an indicator of cell death, membrane lysis, and significant uptake of vital dye (Trypan blue). The obtained results show that after 2 h incubation with different concentrations of lignin, 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.

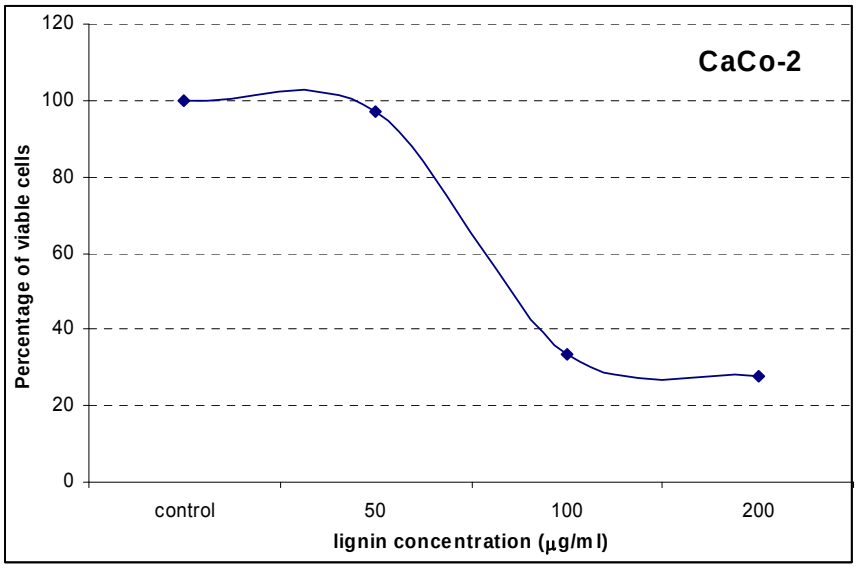

Fig. 2. Percentage of viable cells after treatment with lignin evaluated by the trypan blue exclusion technique in CaCo-2 cells. Values represent the mean of three independent experiments. Statistical in significance (by t-test) as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 3 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 2 h 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.

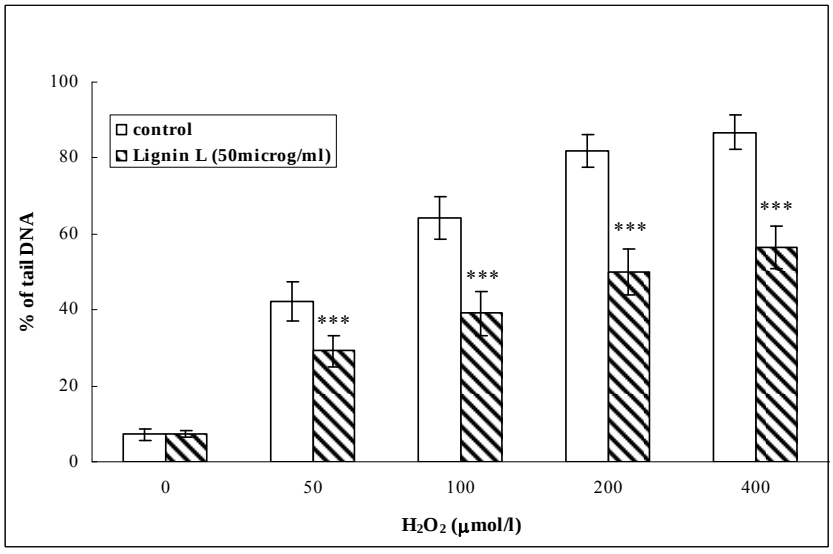

Fig. 3. Decrease in H_2O_2-induced DNA damage in testicular cells from mice by lignin

Figure 4 presents induction of oxidative DNA lesions in visible light-treated testicular cells as well as the protective effect preincubation of cells with lignin.

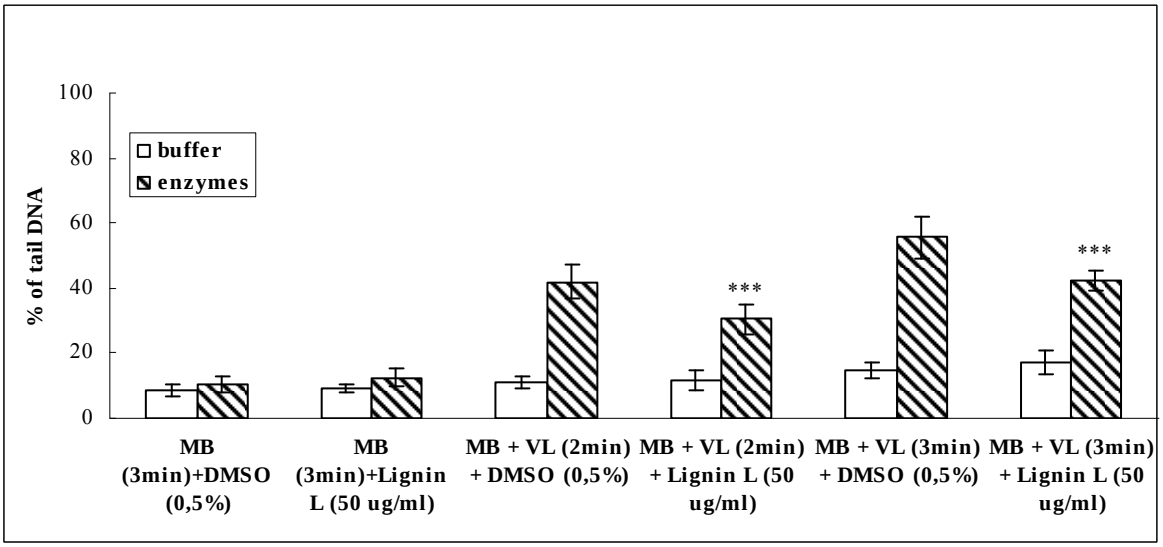

Fig. 4. Decrease in oxidative DNA damage (induced by Methylene blue (MB) + Visible light (VL)) in testicular cells from mice by lignin

Contrary to the effect of hydrogen peroxide, visible light plus MB induced less oxidative DNA lesions in testicular cells. Open bars show the level of direct DNA strand breaks and hatched bars represent oxidative DNA lesions (FPG- and EndoIII-sensitive sites). In further experiments the protective effect of lignin against DNA lesions induced in blood lymphocytes by ·OH radicals was examined. Figure 5 shows that lymphocytes were more sensitive to the effect of hydrogen peroxide than testicular cells.

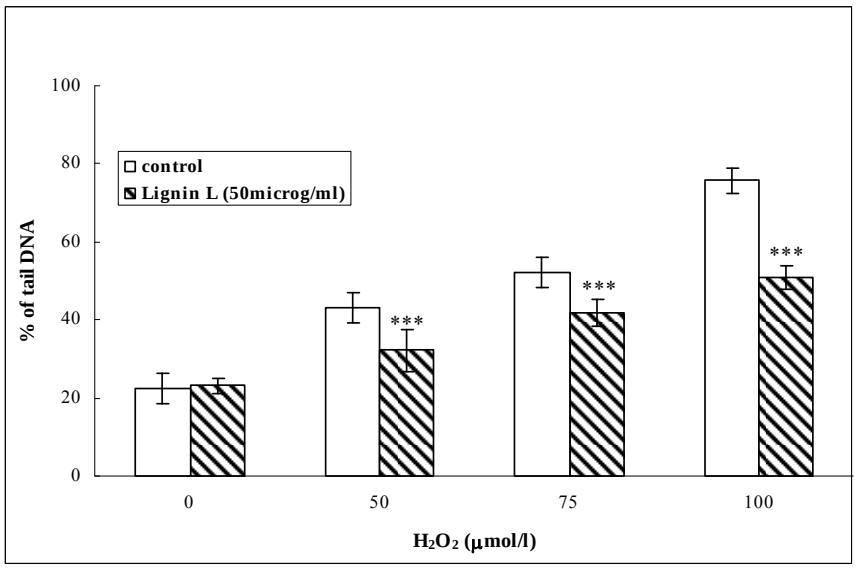

Fig. 5. Decrease in H_2O_2-induced DNA damage in lymphocytes from mice by lignin

Preincubation of lymphocytes *in vitro* with lignin decreased the level of oxidative DNA lesions, when the cells were subsequently treated with visible light – excited MB (Fig. 6).

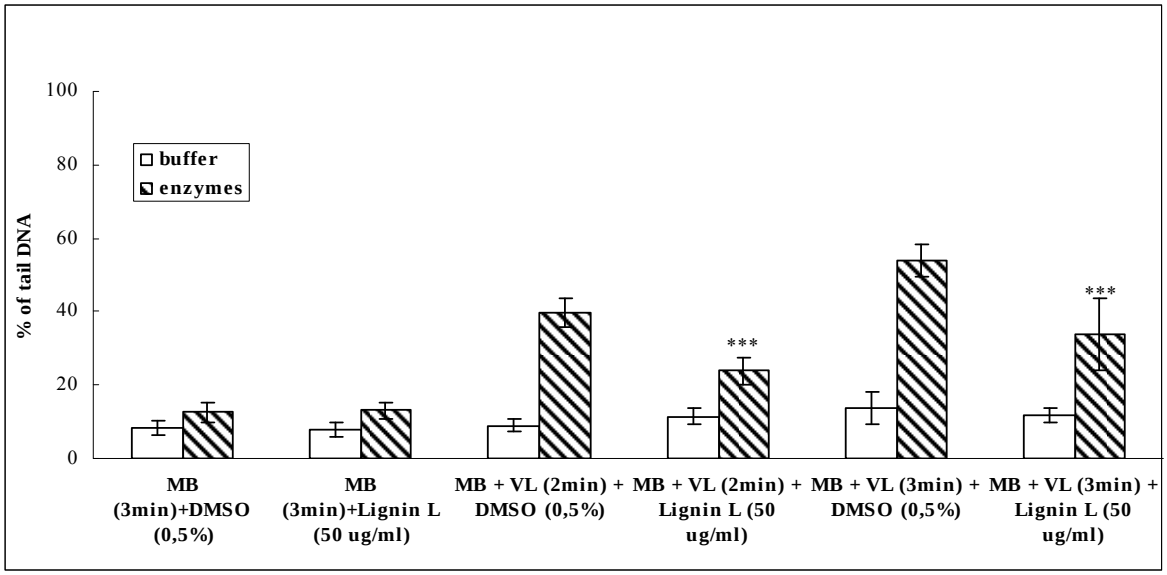

Fig. 6. Decrease in oxidative DNA damage (induced by Methylene blue (MB) + Visible light (VL)) in lymphocytes from mice by lignin.

As it is evident from Figs. 4 and 6, the level of oxidative DNA lesions was decreased at both doses of visible light (120 and 180 s) in both types of cells that were preincubated with lignin. 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 H_2O_2 and visible light was increased. 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 et al. 2004) lignin is partially digested in gut microflora in lignans, which can diffuse into cells and so decrease oxidative damage DNA.

CONCLUSIONS

It can be concluded that phenolic antioxidants could be replaced in multiple processing of polypropylene by lignin, which acts as a radical scavenger due to the presence of unique hindered phenolic hydroxyl groups. The obtained results show that both lignin stabilizers have potential for protection of polypropylene films against degradation during thermo-oxidative aging. They could be incorporated into plastics without health hazard, and they are acceptable for the environment due to their biodegradable nature. The revealed ability of lignin to replace the function of commercial phenolic antioxidants in stabilization of polypropylene matrix towards oxidative degradation is in good correlation with the observed protection effect of lignin against

oxidation damage of DNA in the cells of mice induced by both oxidants hydrogen peroxide and methylene blue (MB) excited by visible light.

The pretreatment of isolated lymphocytes and testicular cells with lignin *in vitro* increased significantly the resistance of both blood lymphocytes and testicular cells to oxidative stress induced by hydrogen peroxide and visible light–excited MB, probably due to consequence of scavenging both hydroxyl radical and singlet oxygen. The observed reduction of oxidative DNA damage indicates that the lignin component of dietary fiber could play a considerable role in the prevention of cancer.

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