Repair of oxidative DNA lesions in blood lymphocytes isolated from Sprague-Dawley rats; the influence of dietary intake of lignin*

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Living organisms possess a variety of self-protective mechanisms which decrease the free radical attack on DNA and so reduce the risk of cancer. Protection of DNA by endogenous antioxidant systems may be significantly increased by numerous exogenously administered antioxidants. Many of them represent important dietary factors. Biopolymer lignin with its phenolic structure can be included into this group of micronutrients. The aim of the present work was to investigate: 1. the effect of biopolymer lignin, given to Sprague-Dawley (SD) rats in diet, on the level of oxidative DNA lesions induced by oxidative stress in freshly isolated peripheral blood lymphocytes *in vitro* and 2. the influence of lignin on kinetics of rejoining of DNA strand breaks induced in lymphocytes under these conditions. As model oxidative agents were used H_2O_2 and visible light in the presence of the photosensitizer Methylene Blue. We found out that dietary intake of lignin caused a significant decrease of H_2O_2-induced DNA strand breaks and visible light-induced oxidative DNA lesions in freshly isolated rat lymphocytes, but it did not influence the kinetics of rejoining of DNA strand breaks.

Key words: hydroxyl radical, singlet oxygen, DNA repair, lignin, rat lymphocytes, single cell gel electrophoresis

Numerous studies have shown that a high intake of micronutrients can decrease the risk of developing cancer. Though while it is by no means clear how this particular diet alters cancer risk, there is substantial metabolic and experimental evidence to implicate antioxidant micronutrients. The simplest scheme of the complex process of carcinogenesis comprises three main steps: initiation, promotion and progression. Oxidative stress may participate in all three steps of carcinogenesis [17]. At the first step, initiation, free radicals activate several mechanisms contributing to changes of genetic information, mutations in the primary structure of DNA which activates an oncogene or inhibits antioncogene (tumor supressor gene). Initiated cells which result from these processes are also influenced by free radicals and exhibit either enhanced proliferation and/or inhibition of cell death. During the final step, free radicals may contribute to uncontrolled growth of tumor cells, genomic instability, and resistance to chemotherapy, invasion and metastasis [17]. Antioxidants are molecules that both directly or indirectly detoxify free radicals of reactive oxygen species (ROS) and thus may protect against cancer. Endogenously produced and exogenously administered antioxidants may act at different steps of carcinogenesis [16]. In this paper we tried to assess the role of the well-known micronutrient, biopolymer lignin in the initiation phase of carcinogenesis. Lignin, together with cellulose and hemicellulose, is a major constituent of the cell wall of plants and the most abundant organic polymer in the biosphere. We examined 1. the ability of this biopolymer to modulate the level of DNA strand breaks produced in peripheral blood lymphocytes by two strong radical oxygen inducers (H_2O_2 and visible light-excited Methylene Blue) and 2. the ability of lignin to influence the kinetics of rejoining of DNA strand breaks induced by one of the oxidative agents, as rejoining of DNA strand breaks is an indicator of DNA repair. While H_2O_2 induced predominantly direct DNA breaks and oxidized DNA bases by $^{\cdot}OH$ radicals [12], visible light in the presence of the photosensitizer Methylene Blue (MB)

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formed oxidized bases via singlet oxygen – $^1O_2$ production [25, 26]. We followed the protective antioxidant activity of lignin, given in food to Sprague-Dawley (SD) rats, against oxidative stress in freshly isolated peripheral blood lymphocytes. Peripheral blood lymphocytes were chosen because dietary lignin or its metabolites circulate in animal organism probably through the blood system. Experiments were performed "*ex vivo*" i.e. lignin was administered to experimental rats which served as a source of lymphocytes treated "*in vitro*", with either H_2O_2 or visible light + MB.

Material and methods

Chemicals. Lignin, which represents a water-soluble sulfur-free lignin preparation of average molecular mass 2,000 was obtained by fractionation of hardwood hydrolysate (170 °C). It contained 19.1% OCH_3 and 0.05% ash. Gel permeation chromatography was performed on a column (53*x*8 cm) of Sephadex LH 60 using a mixture of dioxane and water containing 0.005 mol/l of aqueous NaOH and 0.001 mol/l of LiCl (7:3) as the eluant. Lignin was dissolved in sterile redistilled water and mixed with the pelleted standard control diet (see section "Animals and pre-treatment with lignin").

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 and 100 μmol/l 1 minute before use and kept at 4 °C.

Methylene Blue (MB, product of Loba Feinchemie, Austria) was dissolved in PBS buffer at room temperature in the concentration $3.125x10^{-2}$mol/l. This stock solution was diluted shortly before use in PBS buffer to the final concentration $3.125x10^{-5}$ mol/l and kept at 4 °C.

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 40 mmol/l Hepes-KOH, 0.1 mol/l KCl, 0.5 mmol/l EDTA, 0.2 mg/ml bovine serum albumin, pH=8.0 (1:3000 and 1:1000, respectively) just before use.

Animals and pre-treatment with lignin. Sexually mature male Sprague-Dawley rats were obtained from the firm ANLAB, Czech Republic. The animals (MOL: SD, 180–220 g) were housed two per cage under standard environmental conditions (room temperature 22 ± 2 °C, relative humidity 55 ± 5%, lights on from 06.00 to 18.00 h) in solid plastic cages on hardwood bedding. The rats were fed daily (10 g/100 g body wt/day) (1) apelleted isocaloric standard control diet, which contained nutriment, amino acids, biofactors, and minerals (MP, PD Horné Dubové - Naháč, Slovak Republic), or (2) a lignin-supplemented diet (i.e. 8% of lignin was added to the control diet, approximately 10.4 g/kg/day) for 21 days. The lignin-supplemented diet was prepared by soaking standard pellets in sterile redistilled water with lignin. All rats were given water *ad libitum*. Food consumption for each cage was recorded daily and individual body weights were recorded weekly. After 21 days of feeding, the total body weight gain was 131.3 ± 11.4 g in control rats and 85.8 ± 7.3 g in rats fed with 8% lignin in food. The weight reduction observed in rats fed lignin was not accompanied by any degeneration of inner organs or worsening of the overall health condition.

Isolation of peripheral blood lymphocytes from Sprague-Dawley (SD) rats. Blood lymphocytes were isolated from fresh blood taken directly from the heart of male rats by density centrifugation using Telebrix N300 (Léčivá, Czech Republic). In short, blood was taken from the heart using a heparinized hypodermic needle and carefully arranged in layers on Telebrix N300 (1:1). Telebrix N300 was diluted immediately before use in redistilled water (2:5). After centrifugation (2500 rpm, 30 minutes) the white middle layer containing the lymphocytes was removed, resuspended in PBS buffer and after the next centrifugation (1500 rpm, 10 minutes) the lymphocytes were again resuspended in PBS buffer. The viability of lymphocytes measured by trypan blue exclusion was greater than 95%.

Treatment of lymphocytes with H_2O_2 and visible light (VL) plus MB. Lymphocytes ($3x10^4$) were suspended in 0.75% LMP (low melting point) agarose and spread on a base layer (100 μl 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 50 μl of H_2O_2 solution (concentrations: 50, 75 and 100 μmol/l; 5 minutes at 4 °C) or with visible light (60W bulb; 180 s; 25 cm distance) + MB ($3.125x10^{-5}$ mol/l) on ice without any other source of light. A similar way of light exposure was used by HARTWIG et al [15] in combination with alkaline unwinding. Control cells were treated with MB for 180s only. After treatment the cells were washed with PBS buffer 2 times for 2 minutes.

For observation of repair activity of lymphocytes after H_2O_2 and visible light treatment, the cells were incubated 30, 60 and 120 minutes 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), at 37 °C, 5% CO_2.

Single cell gel electrophoresis (SCGE, Comet Assay). Comet assay is based on the ability of DNA strand breaks 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 [28] was used with minor changes suggested by SLAMEŇOVÁ and co-workers [29] and GÁBELOVÁ and co-workers [11]. Lymphocytes embedded in agarose gels situated on slides were treated with H_2O_2 or visible light + MB. Immediately after treatment (a) the slides with cells (0 min incubation for repair) were placed

in lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, pH=10 and 1% Triton X-100) for 1 hour at 4 °C to remove cellular proteins or (b) the slide with lymphocytes, in which the repair activity was observed, were kept 30, 60 and 120 min in RPMI 1640 medium at 37 °C, 5% CO_2. After incubations, the slides were trasferred to lysis solution too. In the experiments studying the nature and characteristics of H_2O_2- and visible light-induced oxidative DNA damage, we used the modified comet assay suggested by COLLINS and co-workers [4]. After lysis the slides were washed two times for 10 minutes in endonuclease buffer (40 mmol/l HEPES-KOH, 0.1 mol/l KCl, 0.5 mmol/l Na_2EDTA, 0.2 mg/ml BSA, pH=8) and incubated for 30 minutes with a mixture of the enzymes FPG and Endo III at 37 °C. The slides were then transferred to an electrophoresis box containing an alkaline solution at pH 13 (300 mmol/l NaOH, 1 mmol/l Na_2EDTA) and kept in this solution for 40 minutes at 4 °C for DNA strands to unwind. A current of 300 mA (voltage 25 V) was applied for 30 minutes. The slides were removed, neutralized by 35 minute washing in Tris-HCl (0.4 mol/l, pH=7.5), stained with 20 μl ethidium bromide (EtBr, 5 μg/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, Kineting Imaging, Liverpool, UK) for determination of DNA in the tail, linearly related to the frequency of DNA strand breaks [23].

Statistical analyses. For comparison of the level of DNA strand breaks induced in blood lymphocytes isolated from rats fed common diet (C rats) or lignin-supplemented diet (L rats) at time 0 at each concentration of H_2O_2 and for comparison of DNA strand breaks between samples of both categories at times 30 and 60 min at equal concentrations of H_2O_2 so called 2-samples 2-sided Student's t-test was used. For comparison of the level of VL+MB-induced oxidative DNA lesions between blood lymphocytes of both categories at time 0, 30, 60 and 120 min Student's t-test was also used. For comparison of VL+MB-induced oxidative DNA damages between individual times of incubation in lymphocytes from L rats and between individual times of incubation in lymphocytes from C rats so called "One way analysis of variance test" and "Least significant difference (LSD) test" was used.

Results

Figure 1 presents the level of direct DNA strand breaks in H_2O_2 (50, 75, and 100 μmol/l)-treated blood lymphocytes isolated from C rats – left side and from L rats – right side). Moreover, Figure 1 shows reduction of these DNA lesions after 30 and 60 min post-incubation of lymphocytes in RPMI 1640 medium at 37 °C and 5% CO_2 (rejoining of H_2O_2- induced single strand DNA breaks). Induction of

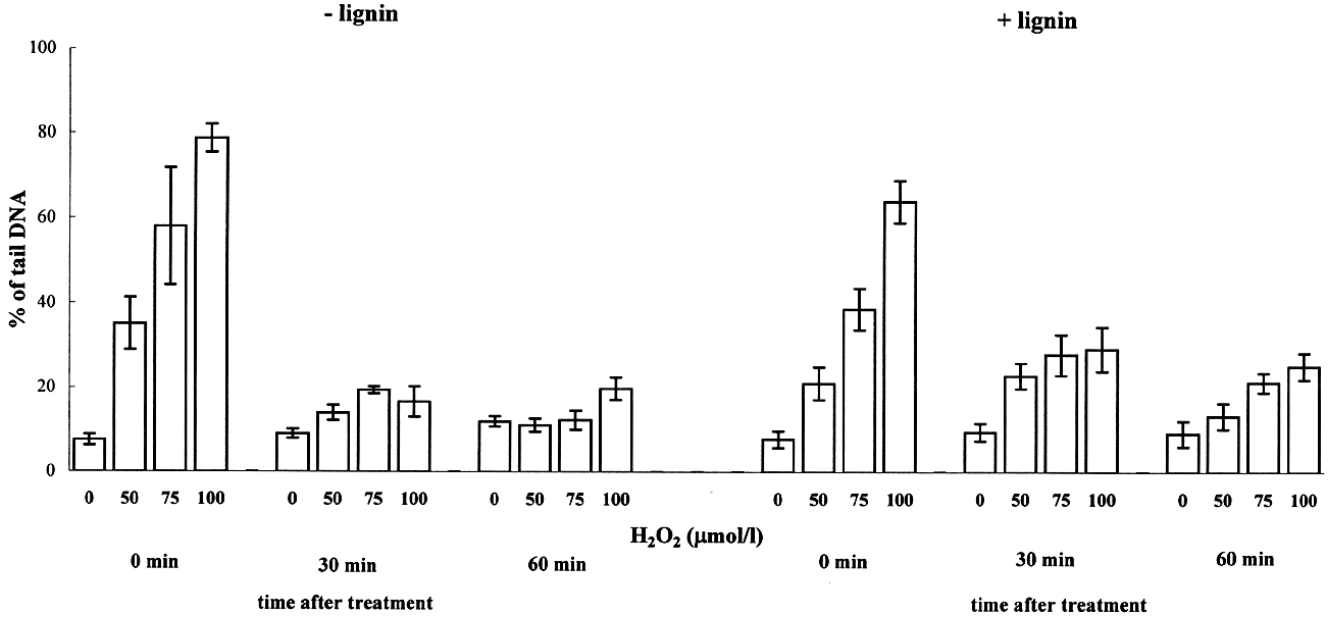

Figure 1. Levels of induced single strand DNA breaks in H_2O_2-treated lymphocytes isolated from control rats (C rats) (left side) and rats fed lignin-supplemented diet (L rats) (right side) immediately after treatment (0 min) and during incubation after treatment (30 min and 60 min). Data represent the mean of two independent experiments (with four parallels each) ± standard deviation.

Student's t-test showed that at time 0 min were the levels of DNA strand breaks in lymphocytes from C rats significantly higher at concentrations 50 (p=0.002), and 100 (p=0.005) μmol H_2O_2 than in lymphocytes from L rats; at time 30 min was in group of lymphocytes from C rats (treated with 0, 50, 75, 100 μmol/l of H_2O_2) level of DNA strand breaks was significantly lower than in lymphocytes from L rats and at time 60 min there were not any significant differences between lymphocytes from C rats and L rats.

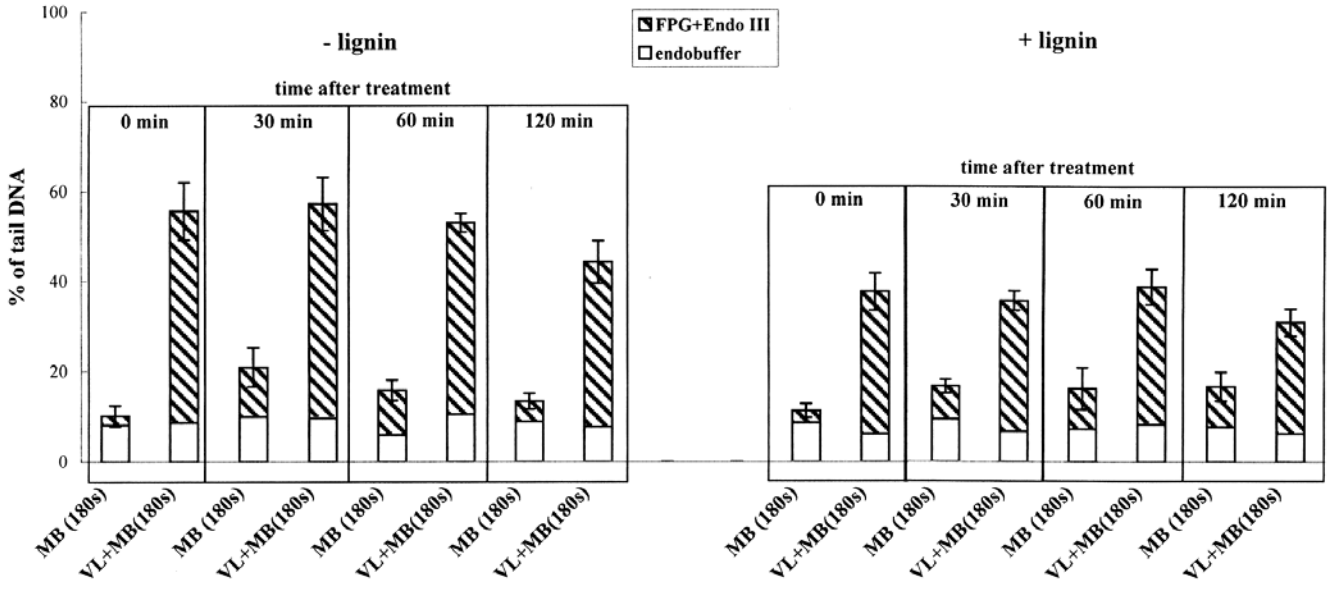

Figure 2. Levels of induced oxidative DNA lesions in Methylene Blue (MB)- or visible light excited Methylene Blue (VL+MB)-treated lymphocytes isolated from control rats (C rats) (left side) and rats fed lignin-supplemented diet (L rats) (right side) immediately after treatment (0 min) and during incubation after treatment (30, 60 and 120 min). Data represent the mean of two independent experiments (with four parallels each) ± standard deviation. Open parts of bars represent the level of direct DNA strand breaks and hatched parts of bars represent oxidative DNA lesions (FPG+Endo III sensitive sites).
Student's t-test showed that the levels of oxidative DNA damages in lymphocytes from C rats were significantly higher at time 0 min (p=0.003), 30 min (p<0.001), 60 min (p=0.001) and 120 (p=0.005) incubation after treatment than in lymphocytes from L rats. One way analysis of variance test and Least significant difference (LSD) test showed statistical decrease of (VL+MB)-induced oxidative DNA damages with increasing time of incubation only in lymphocytes from C rats (between times: 0 and 120 min – p=0.019; 30 and 120 min – p=0.009; 60 and 120 min – p=0.031).

direct DNA strand breaks has increased proportionally to the concentration of H_2O_2 in both kinds of lymphocytes; however, at each concentration of H_2O_2 the level of DNA damage was significantly lower in lymphocytes isolated from L rats than in lymphocytes isolated from C rats. Post-H_2O_2-incubation of lymphocytes from C rats for 30 min was sufficient for rejoining of almost all DNA lesions induced by H_2O_2 and following 30 min incubation of these cells did not manifest any further effect. In lymphocytes isolated from L rats was not rejoining of H_2O_2-induced strand breaks so efficient than in lymphocytes from C rats during the first 30 min of incubation. A statistically significant decrease of DNA damage induced by each concentration of H_2O_2 was observed in lymphocytes from L rats only after 60 min of post-H_2O_2-incubation.

Figure 2 presents direct single strand DNA breaks (open bars) and oxidative DNA damages (hatched bars) induced by visible light (VL, 180s) in the presence of photosensitizer Methylene Blue (MB) in C rats and L rats. Oxidative damages represented FPG and Endo III sensitive sites of DNA after MB and/or VL+MB treatment. Figure 2 points out also the reduced level of oxidative DNA lesions after 30, 60, and 120 min incubation of lymphocytes in RPMI 1640 medium at 37 °C and 5% CO_2, indicating DNA repair. Direct DNA strand breaks and oxidative DNA damages of lymphocytes isolated from C rats as well as in lymphocytes isolated from L rats were approximately equal after treatment with MB alone. 180s influence of VL+MB on lymphocytes isolated from both, C rats and L rats significantly increased the level of oxidative DNA damage but the level of oxidative DNA lesions of lymphocytes from L rats was significantly lower than in lymphocytes from C rats. 30 and 60 min incubation of lymphocytes isolated from C rats in RPMI medium was not efficient; we did not observe any repair of oxidative DNA damages. For repair of oxidative lesions in lymphocytes from C rats was inevitable 120 min long incubation, although a substantial level of non-repaired lesions has remained. In the case of lymphocytes isolated from L rats 120 min incubation after treatment with VL+MB was not sufficiently long for achieving repair of VL+MB induced oxidative DNA lesions.

Discussion

Molecular oxygen is relatively nonreactive and essential for cells, but during metabolism or after exposure to exogenous factors (ionising radiation or chemicals) it induces formation of reactive oxygen species (ROS) [10]. ROS include not only free radicals (hydroxyl radical, $^{\cdot}OH$, and superoxide radical, $O_2^{\cdot-}$), but also molecules such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3); they are able to attack molecules DNA directly or indirectly [2]. The genotoxic effect of hydrogen peroxide to DNA of

cells is mediated mainly by highly reactive oxidant $^{\cdot}OH$ generated by the reaction of reduced transition metals (Fe^{2+} or Cu^{2+} ions) with H_2O_2 via Fenton reactions [12]. It was shown that DNA damage profile induced by $^{\cdot}OH$ consists of approximately equal levels of oxidized DNA bases, abasic sites, and strand breaks [8]. Treatment of rat lymphocytes with H_2O_2 led to very clear dose-dependent increase of direct DNA strand breaks with very rapid DNA rejoining (Fig. 1, left side). Within 30 min incubation after treatment the level of strand breaks has returned nearly to the background control level. From this point of view it is interesting that in human lymphocytes takes rejoining of H_2O_2-induced DNA strand breaks much longer time [6, 7].

Visible light in presence of photosensitizers induces oxidative DNA damages either via singlet oxygen – $^1O_2$ (type II reaction), or excited molecules of photosensitizers react with DNA directly (type I reaction) [25, 26]. Methylene Blue belongs among type II photosensitizers. DNA damages induced by $^1O_2$ are oxidative alterations to guanine residues, which are sensitive to several site-specific DNA glycosylases (among them the bacterial FPG protein). Promutagenic DNA adduct 7,8-dihydro-8-oxoguanine predominates [3]. Oxidative DNA lesions can be identified by the modified comet assay [4]. Unlike hydrogene peroxide, treatment of rat lymphocytes with visible light did not lead to formation of DNA strand breaks (Fig. 2, left side). This is in correlation with findings of several authors who reported that visible light induced in the presence of photosensitizer much higher excess of DNA base modifications in comparison with DNA strand breaks [8, 25]. DNA damages induced by visible light represent oxidized modifications (predominantly oxidized purines), which can be revealed after incubation of treated cells with specific DNA repair endonucleases FPG and Endo III and detected as strand breaks using the comet assay. We found out that repair of oxidative DNA lesions was in rat lymphocytes very slow; 120 min long incubation was inevitable, although a substantial level of non-repaired lesions remained (Fig. 2, left side).

Numerous studies indicate that supplementation of human or animal diet with various dietary fibers (DF) can protect the organism against development of cancer. LU et al [22] have suggested that the lignin content, which is one of the important components of DF (plant cell walls contribute more than 95% of DF [14]), plays a crucial role in this protection. Lignin has some medicinal importance because it has an anti-immunodeficiency virus and anti-influenza virus activity [13, 24] and is an effective binder of bioacid products of cholesterol degradation [18] and nitrosamines [19]. The adsorptive ability of lignin is supplemented with its antioxidant activity owing to the presence of unique hindered phenolic hydroxyl groups, which act as a stabilizer of reactions induced by oxygen and its radical reduction products [22, 30]. This dual effect of lignin in *in vitro* conditions was confirmed in several papers [18, 20, 21, 31], we therefore tried to ascertain the effects of lignin *in vivo*. As administration of lignin in food is a natural route which could be applicable to humans, we used for *in vivo* application of lignin rats which fed lignin-supplemented diet for 21 days. These animals served as a source of peripheral blood lymphocytes, which were then treated with one of the oxidative agents. As it is evident from the Figure 1 (right side) and Figure 2 (right side), the level DNA strand breaks induced after H_2O_2 treatment and oxidative DNA damages induced by visible light in the presence of Methylene Blue was significantly lower than in lymphocytes from control animals (C rats), what means that lignin protects DNA against damaging effects of studied oxidative agents.

The most frequent oxidative DNA lesions in mammalian genomes are removed by the base excision repair (BER) via multiple pathways that involve the replacement of one or more nucleotides at the lesion site, though it was shown that also nucleotide and transcription-coupled DNA repair pathways could be involved (reviewed in [27]). The biological consequences of a BER defect are at present largely unknown. FORTINI et al [9] reported that in mouse cells defective in the main BER DNA polymerase beta (Pol beta) seems to be dispensable for repair of single strand breaks induced by hydrogen peroxide. As DNA repair has animportant influence on DNA stability and ultimately cancer incidence, we tried to ascertain if lignin-supplemented diet could influence DNA repair in lymphocytes treated by hydrogen peroxide or visible light. There are increasing numbers of studies which suggest that the diet might have an affect on DNA repair capacity in humans. For example, COLLINS and co-workers have reported, that DNA repair in human lymphocytes was positively stimulated by consumption of kiwifruit [5]; according to ASTLEY et al [1], dietary supplementation with carotenoids and carotenoid-rich foods can influence repair of DNA damage in human lymphocytes by modulation of discrete stages in the DNA repair mechanisms. As rejoining of single strand DNA breaks is considered an indicator of DNA repair, we followed the kinetics of rejoining of single strand DNA breaks in lymphocytes isolated from C rats and L rats, after the treatment with H_2O_2 or visible light+MB. Figure 1 shows, that the rejoining of DNA strand breaks in H_2O_2-treated lymphocytes from L rats was slower than in lymphocytes from C rats. DNA repair of oxidative DNA lesions induced by visible light + MB (Fig. 2) was not detected in lymphocytes from L rats at all. We suggest that visible light + MB-treated lymphocytes evidently need more extended period of incubation (more than 120 min) for repair oxidative DNA damages. Similar conclusions were presented by LÁBAJ et al [21], who did not observe any stimulating influence of lignin to the kinetics of DNA rejoining in MNNG-treated V79 cells *in vitro*.

We can conclude, that though a lignin-supplemented diet protected DNA of blood lymphocytes isolated from SD rats

against DNA damage induced by oxidative stress, it failed to influence the kinetics of DNA rejoining, which is an indicator of DNA repair.

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