Diet containing fungal (1→3)-β-D-glucan derivative exhibits protective effects against DNA lesions induced in freshly isolated rat cells*

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Dietary effect of water-soluble derivative – carboxymethyl chitin-glucan (CM-CG) on the level of DNA lesions induced by hydrogen peroxide (H_2O_2) was examined in *ex vivo* experiments. Lymphocytes, testicular cells, alveolar macrophages and epithelial II cells were isolated from Sprague Dawley rats fed a common or CM-CG enriched diet (200 mg/kg of body weight) during 21 days. Freshly isolated cells were in *in vitro* conditions exposed to H_2O_2 and the levels of DNA breaks were evaluated by single cell gel electrophoresis.

A dose-dependent increase of DNA breaks was observed after treatment with hydrogen peroxide in all studied cell types. The levels of DNA breaks in cells isolated from CM-CG supplemented animals were lower compared to the levels of DNA breaks in cells isolated from animals fed a common diet.

Intake of CM-CG enriched diet did not increase the level of DNA damage in different kinds of freshly isolated rat cells and equipped the cells with resistance to the treatment with hydrogen peroxide.

Key words: carboxymethyl chitin-glucan, hydrogen peroxide, single cell gel electrophoresis, Sprague Dawley rats

β-glucans, or polymers of D-glucose linked by (1→3)-β and (1→6)-β glycosidic linkages represent the major constituent of the fungal cell wall that defines its shape and rigidity. Pharmacologically they are classified as biological response modifiers because they stimulate the host's immune system. This enhancement results in antitumor effect and metastasis inhibition, antibacterial, antiviral, anticoagulatory and wound healing activities, as well as in stimulation of hematopoiesis and radioprotection [3].

Since fungal β-glucans are due to their high molecular weight as well as to interchain associations poorly soluble in aqueous media, their clinical application is limited. There are several approaches to achieve better solubility and increased biological activity, like hydrolysis, ultrasonication or introduction of the charged groups in the glucan molecule, for example carboxymethyl or sulfoethyl group (reviewed in [16]).

While there is a wealth of knowledge regarding the β-glucans activities and their obvious therapeutic potential, there are also some investigations that have been carried out to study the possible antimutagenic effect of β-glucans. PATCHEN et al [21], SAKAGAMI et al [24], BABINCOVÁ et al [2], KRIŽKOVÁ et al [17], SLAMEŇOVÁ et al [26] have reported free radical scavenging activity for β-glucan or β-glucan-containing products. Anti-clastogenic effect of β-glucan has been extensively studied by CHORVATOVIČOVÁ et al [5, 6, 7, 8, 9, 10] and TOHAMY et a. [30]. They also confirmed the protective effect of β-glucan treatment. On the other hand, GÁBELOVÁ and PLEŠKOVÁ [13] did not observe after the short-term pre-incubation of human colonic cells Caco-2 with β-glucan derivative any reduction of the oxidative DNA damage and also TSIAPALI et al [32] described weak antioxidant activity of β-glucans.

The objective of our study was to examine in *ex vivo* experiments the dietary effect of water-soluble derivative – carboxymethyl chitin-glucan (CM-CG) on the level of DNA lesions induced by hydrogen peroxide (H_2O_2). Lymphocytes, testicular cells, alveolar macrophages and epithelial II cells were isolated from Sprague Dawley rats fed a common or CM-CG enriched diet (200 mg/kg of body weight) during 21 days. Freshly isolated cells were in *in vitro* conditions exposed to H_2O_2 and the levels of DNA breaks were evaluated by single cell gel electrophoresis.

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Material and methods

Animals. Male Sprague Dawley (SD) rats with a mean weight of 200 g (range, 185–210 g) were used. 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 $55 \pm 5\%$ and housed in plastic cages on hardwood bedding (2 animals per cage). They were given free access to tap water and standard diet (MP, PD Horné Dubové - Naháč, Slovak Republic) or CM-CG enriched diet which was prepared by soaking standard pellets in sterile redistilled water with CM-CG for 21 days. 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 ~ 125 g (control rats), respectively ~ 110 g (rats feeding CM-CG food). In rats fed CM-CG any degeneration of inner organs or worsening of the overall health condition was not observed.

Chemicals. The chitin-glucan complex was isolated from the cell walls of the filamentous fungus *Aspergillus niger*, an industrial strain used for the commercial production of citric acid. Water soluble derivative carboxymethyl chitin-glucan (CM-CG) was prepared according to the procedure described by MACHOVÁ et al [19] and was obtained from Dr. G. Kogan (Institute of Chemistry, SAS, Slovak Republic).

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 and 200 μmol/l shortly before use and kept at 4 °C. The cells embedded in agarose on microscope slides were treated with 50 μl of appropriate concentration of H_2O_2 and cells plated in 24-wells plates with 500 μl of H_2O_2 for 5 min on ice in the dark.

Isolation of the cells. Rat testicular cells were isolated from the rat testes of sexually mature male SD rats by enzymatic digestion as described by BRADLEY and DYSART [4], with some modifications [28]. 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%.

Blood lymphocytes were isolated from the blood taken by cardial punction followed by density centrifugation using Telebrix N300 (Léčiva, Czech Republic). Briefly, blood was taken from the heart using a heparinized hypodermic needle and carefully layered on Telebrix N300 (1:1). Telebrix N300 was diluted immediately before use in redistilled water (2:5). After centrifugation (2500 rpm, 30 min) the white middle layer containing the lymphocytes was removed, resuspended in PBS buffer and after the next centrifugation (1500 rpm, 10 min) the lymphocytes were processed by comet assay. The viability of lymphocytes measured by trypan blue exclusion was greater than 95%.

A population of alveolar macrophages and epithelial II cells isolated from rat lung, according to the method of MYRVIK et al [20] and RICHARDS et al [23], HOET et al [15], respectively. The procedure includes lung perfusion, lung lavage, trypsin digestion, Percoll gradient centrifugation and differential adherence. Briefly, the rats were intraperitoneally anesthetized with sodium pentobarbital (60 mg/kg). The trachea was cannulated, the lungs were perfused with 0.9% NaCl via pulmonary artery and mechanically ventilated. The lungs were excised, lavaged via the trachea with 0.9% NaCl (5 times with 5 ml). The alveolar macrophages were obtained by centrifugation of bronchoalveolar lavage fluid (10 min, 1600 rpm) and the pellet was suspended in complete DMEM. The lung tissue was trypsinized (175 mg of trypsin in 75 ml of PBS with calcium and magnesium) during 30 min at 37 °C. The lungs were chopped and 5 ml of FBS and 2.5 mg of DNase I were added. After shaking and filtering, the cell suspension was layered onto Percoll gradient and centrifuged for 20 min at 1600 rpm. The cells from the layer above the heavy gradient were removed, resuspended in PBS and 1 mg of DNase I was added. After centrifugation (10 min, 1600 rpm) the pellet was suspended in serum free DMEM and plated in a 60-mm diameter culture dish for 1 h incubation (5% CO_2, 37 °C) to let cells other than pneumocytes attach. The unattached cells were suspended in DMEM supplemented with 10% FBS, 200 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin. The cell viability assessed by trypan blue exclusion was greater than 95%.

Epithelial II cells and alveolar macrophages were plated at a density of 5×10^5 cells/well in 24-wells plastic plates and incubated at 37 °C in an atmosphere of 5% CO_2 for 20 h.

Growth media and other chemicals used for cell cultivation were purchased from GIBCO.

Single cell gel electrophoresis. The procedure of SINGH et al [25] was used with minor modifications suggested by SLAMEŇOVÁ et al [26] and GÁBELOVÁ et al [14]. Briefly, 2×10^4 cells (in 85 μl of 0.75% low-melting agarose) were spread on a base layer of 100 μl of 1% normal-melting agarose placed on microscope slide and covered with a cover slip. After solidification of the gel, the cover slip was removed. Lymphocytes and testicular cells were embedded in agarose and then exposed to H_2O_2, alveolar macrophages and epithelial II cells were treated in plastic plates. The slides were then 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 h at 4 °C to remove cellular proteins. After lysis the

slides were transferred to an electrophoresis buffer (300 mmol/l NaOH, 1 mmol/l Na_2EDTA, 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 (0.4 mol, pH 7.5) three times for 5 min and stained with ethidium bromide (EtBr, 10 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.

Statistics. The results represent a mean from 2 sets of experiments. In each experiment, 2 animals were used as a control and 2 animals as a treated group. From each concentration of hydrogen peroxide, 3 parallel slides were made in one experiment. The significance of differences between samples was evaluated by Student's t-test (statistically decreased from untreated samples – "common diet" – $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

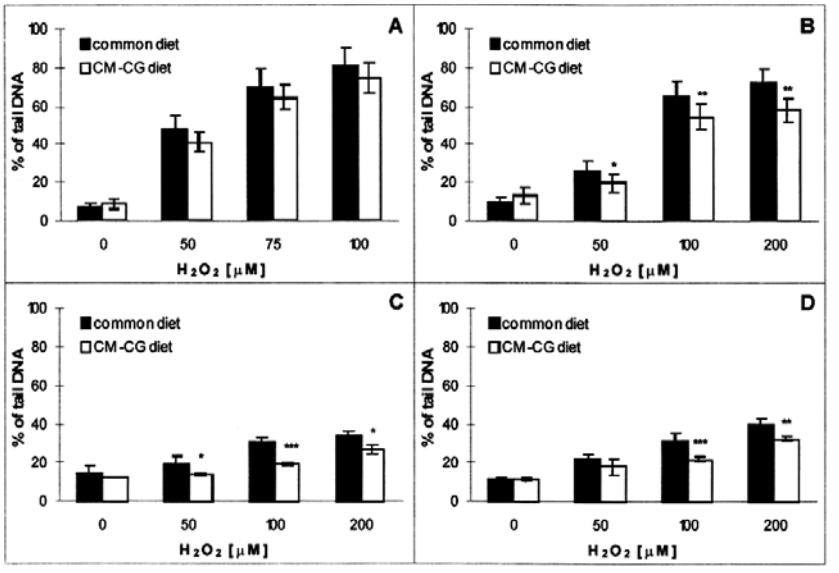

Figure 1. Level of DNA breaks after 5 min treatment with hydrogen peroxide (50–200 μM) in lymphocytes (A), testicular cells (B), alveolar macrophages (C) and epithelial II cells (D) isolated from rats fed either a common (filled bars) or CM-CG supplemented diet (open bars). Asterisks show significant difference from untreated sample ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$). Data represent the mean of two independent experiments (with six parallels each) + standard deviation.

Results

Figure 1A represents DNA damage induced by 5 min treatment with hydrogen peroxide (50, 75, 100 μM) in lymphocytes isolated from rats fed either a common (filled bars) or CM-CG supplemented diet (open bars). A dose-dependent increase of DNA breaks was obvious in both cases; but the levels of DNA breaks in cells isolated from CM-CG supplemented animals were lower compared to the levels of DNA breaks in cells isolated from animals fed a common diet.

The protective effect of CM-CG was detected also in testicular cells, as shown in Figure 1B. DNA breaks were induced by treatment with higher concentrations of hydrogen peroxide (50, 100 and 200 μM). The level of DNA damage in testicular cells isolated from animals fed CM-CG enriched diet (open bars) did not reach values of DNA damage in cells isolated from animals fed a common diet (filled bars). This significant decrease of DNA breaks was observed in all used concentrations ($^{*}p<0.05$, $^{**}p<0.01$).

Figure 1C shows the percentage of tail DNA induced by hydrogen peroxide (50, 100 and 200 μM) in alveolar macrophages isolated from rats fed either a common (filled bars) or CM-CG supplemented diet (open bars). A concentration-dependent increase of DNA breaks was evident in both cases and again the level of DNA breaks in macrophages isolated from CM-CG fed animals was significantly lower compared to the level of DNA breaks in macrophages isolated from rats fed a common diet ($^{*}p<0.05$, $^{***}p<0.001$).

Figure 1D shows the level of DNA breaks induced by hydrogen peroxide (50, 100 and 200 μM) in epithelial II cells. Filled bars represent cells isolated from rats fed a common diet and open bars represent cells from CM-CG supplemented animals. Again, the dose-dependent increase of DNA breaks was apparent in both cases and pneumocytes isolated from CM-CG supplemented animals were more resistant to hydrogen peroxide ($^{**}p<0.01$, $^{***}p<0.001$).

Table 1 summarizes the levels of DNA damage measured by comet assay in all used cell types, as presented in Figure 1. We can conclude that the intake of CM-CG enriched diet did not increase on the level of DNA damage in different kinds of freshly isolated rat cells and these cells were more resistant to the treatment with hydrogen peroxide.

Discussion

In recent years extensive studies have been carried out to study the properties of yeast polysaccharides, mainly the immunomodulatory effects of β-glucans. One of the mechanisms which may explain the chemopreventive and radioprotective effect of β-glucans is their ability to trap free radicals [6, 8, 21]. Reactive oxygen species are generated endogenously during metabolism and are produced as well by various environmental stimuli [12]. As the oxidative da-

Table 1. Level of DNA breaks induced by hydrogen peroxide in different kinds of freshly isolated rat cells

Cell type	Diet	Percentage of tail DNA in H_2O_2 treated cells				
		0 μM H_2O_2	50 μM H_2O_2	75 μM H_2O_2	100 μM H_2O_2	200 μM H_2O_2
lymphocytes	common	7.16	48.3	70.06	80.85	–
	CM-CG enriched	8.91	41.17	64.2	74.42	–
testicular cells	common	9.78	26.61	–	65.01	71.55
	CM-CG enriched	13.28	19.74*	–	54.64**	58.1**
macrophages	common	14.64	19.56	–	30.06	34.055
	CM-CG enriched	12.06	13.88*	–	19.21***	26.94*
pneumocytes	common	10.99	22.36	–	31.59	39.99
	CM-CG enriched	11.22	18.26	–	21.83***	32.38**

Comparison of the levels of DNA breaks in hydrogen peroxide treated lymphocytes, testicular cells, alveolar macrophages and pneumocytes isolated from control or CM-CG fed rats. Asterisks show significant difference from untreated sample ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

mage of biomolecules, especially DNA, means threat to organism, and may cause degenerative processes such as aging or cancer [1, 18]; an antioxidant protection has attracted considerable interest during the last decade.

In order to evaluate the possible protective effect of carboxymethyl chitin-glucan (CM-CG), we studied its effect on the level of DNA breaks induced by hydrogen peroxide in different kinds of freshly isolated rat cells. Blood lymphocytes, testicular cells, alveolar macrophages and epithelial II cells were isolated from animals fed either a common or CM-CG enriched diet for 21 days (200 mg of CM-CG/kg of body weight). Cells were then challenged *ex vivo* with various concentrations of hydrogen peroxide.

Hydrogen peroxide is a well-known oxidant agent which via $^{\cdot}OH$ radicals attacks DNA and causes fragmentation of DNA, base modification or even base loss, DNA strand breaks [22]. Highly reactive $^{\cdot}OH$ radicals are generated within the cells during the Fenton (or Haber-Weiss) reaction in which hydrogen peroxide reacts with reduced transition metal ions (Fe^{2+}, Cu^{+}). Direct DNA strand breaks induced by hydrogen peroxide are rejoined very fast, but DNA damage persists in the form of oxidized bases longer [11, 14]. Treatment of the freshly isolated rat cells with hydrogen peroxide led to dose-dependent increase of DNA breaks. As seen in the figures (Fig. 1A–D), DNA damage in all cell types isolated from animals fed CM-CG supplemented diet did not reach the values as did DNA damage in cells isolated from animals fed a common diet.

It has been shown that administration of the insoluble glucans by parenteral routes was associated with toxicity which manifested itself as hepatosplenomegaly, granuloma formation, micro-embolization and enhanced endotoxin sensitivity [33]. For this reason, the oral application of water-soluble β-glucans derivatives would have advantage of easy administration without any harmful side-effects. It has been often stated that pure polysaccharides cannot be absorbed from digestive tract so parenteral route is required to achieve immunomodulatory effect [31]. In contrast, SUZUKI et al [29] have reported enhancement of the immune response after oral administration of SSG, a β-glucan obtained from fungus *Sclerotinia sclerotiorum.*

CHORVATOVIČOVÁ and ŠANDULA [9] studied antimutagenic effect of CM-CG by comparing different routes of administration, intraperitoneal, intravenous and oral. They confirmed beneficial effect of CM-CG after intraperitoneal and intravenous administration, oral application of CM-CG did not exert an anticlastogenic effect. They used high molecular CM-CG (600 kDa) therefore it is conceivable that CM-CG failed to pass through the gastrointestinal tract. We used the same dose of CM-CG (200 mg/kg of body weight) as CHORVATOVIČOVÁ and ŠANDULA [9], with a different scheme of experiments. Animals were pretreated with multiple doses of CM-CG for 21 days; control animals fed a common diet. CM-CG itself did not induce any negative effect on the level of DNA breaks in rat cells; on the contrary, the cells isolated from CM-CG fed animals were more resistant to hydrogen peroxide (Tab. 1). Molecular weight of CM-CG used in our experiments was 277 kDa so we assumed that CM-CG passed through gastrointestinal tract and manifested its protective effect detected in different cell types. CHORVATOVIČOVÁ et al [7] proved in futher experiments with ultrasonically lowered molecular weight of carboxymethyl chitin-glucan its ability to pass through the wall of the gastrointestinal tract. Our data are also in concordance with previous findings of SLAMEŇOVÁ et al [27] and KRIŽKOVÁ et al [17] where CM-CG exhibited its scavenging and absorptive ability and thus protected DNA.

On the base of our results we can conclude that the study of antimutagenic effects of orally administrated glucans with low molecular weight could present a way, how to achieve effective constituent with a broad range of benefits for humans.

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