Carboxymethyl chitin-glucan enriched diet exhibits protective effects against oxidative DNA damage induced in freshly isolated rat cells*

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The connection between dietary intake of carboxymethyl chitin-glucan (CM-CG, approximately 200 mg/kg body weight, during 21 days) and the response of freshly isolated rat cells to genotoxic treatment with a combination of photosensitizer Methylene Blue and visible light (MB+VL) was evaluated in presented study. Blood lymphocytes, testicular cells, and hepatocytes were isolated from rats fed by a standard or CM-CG enriched diet and in *ex vivo* conditions challenged with oxidative agent. Induced DNA damage was assessed using a modified comet assay.

When added to the diet, CM-CG itself did not induce any negative effect on the health condition of animals or on level of DNA breaks in rat cells. Moreover, the cells isolated from CM-CG fed animals were more resistant to oxidative stress induced by visible light-excited Methylene Blue.

In conclusion, we have demonstrated that carboxymethyl chitin-glucan represents a natural fungal polysaccharide that is able to exert antimutagenic properties upon application in diet.

Key words: photosensitization, oxidative DNA lesions, carboxymethyl chitin-glucan, diet, comet assay

Photosensitization occurs due to the interaction of visible light with a sensitizer in the presence of oxygen and gives rise to a highly effective reactive oxygen species (ROS), capable to exert damage in various biological systems [1]. Exposure to ROS of either endogenous or exogenous origin contributes to oxidative stress, which is linked to degenerative processes, such as aging, as well as to etiology of several chronic diseases including coronary heart disease, arthritis, or cancer [2–4]. Cellular targets for oxidative modifications include all types of cell components including DNA, presumably the most critical target. Thus, search for compounds capable of minimizing oxidative damage, is one of the strategies that may lead to control of the degenerative diseases. One of the target groups of such compounds are fungal polysaccharides.

Polysaccharides chitin and β-D-glucan are present in the mycelium of a filamentous fungus *Aspergillus niger*, being covalently linked into a complex and it can be assumed that both of them could be responsible for protective effect exerted by the water-soluble derivative of this complex [5], since a broad range of biological activities of these carbohydrates has been previously described in many studies [reviewed in 6–8]. A water-soluble derivative, carboxymethyl chitin-glucan (CM-CG) used in our study enables oral administration without detrimental side-effects associated with administration of the insoluble fungal β-D-glucans [9, 10]. Protective effects of this derivative against DNA damage have been already described [11–16], however the precise mechanism of the action remains still unclear. Several authors suggested that the antimutagenic effect of β-D-glucans might be explained by their free radical scavenging activity [17–20]. On the other hand, also failure of β-D-glucans to exert significant antioxidant or free radical scavenging activity has been described [21, 22].

The aim of the present study was therefore to establish the effects of dietary CM-CG on the level of oxidative DNA lesions induced by visible light (>400 nm) excited photosensitizer Methylene Blue in freshly isolated rat cells (lym-

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phocytes, testicular cells, and hepatocytes) in *ex vivo* experiments. After 21 days of feeding with a standard or CM-CG enriched diet, the cells were isolated, challenged with oxidative agent, and the induced DNA damage was assessed using a modified comet assay, which enables detection of oxidative modifications of DNA bases.

Material and methods

Animals. Eight male Sprague Dawley (SD) rats with a mean weight of 200 g (range, 180–220 g) were used. Four animals comprised a control group fed with a standard diet and four animals received CM-CG supplemented food. The animals were obtained from an in-house strain and were maintained in a room with 12 h light/dark cycle, room temperature 22±2 °C, relative humidity 55+5% and housed in plastic cages on hardwood bedding (2 animals per cage). Water was provided *ad libitum*, and standard diet (containing nutrients, amino acids, biofactors, and minerals) or CM-CG enriched diet, which was prepared daily by soaking standard pellets in sterile redistilled water containing CM-CG (200 mg/kg body weight) was given. About 25 g of standard diet per animal was soaked in approximately 15 ml of water containing 2.5–3.0 mg CM-CG. Afterwards, the soaked mixture was pelleted, air dried, and used for feeding the animals. 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 ~120 g (control rats) or ~110 g (rats fed with CM-CG supplemented diet). In rats receiving CM-CG containing diet, no degeneration of inner organs or worsening of the overall health condition was 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 (Biopo, Leopoldov, Slovak Republic). The freeze-dried samples contained 2.24% nitrogen, which corresponds to a chitin content of about 30%. Water soluble derivative carboxymethyl chitin-glucan (CM-CG) was prepared according to the procedure described by MACHOVÁ et al [23]. The degree of substitution (DS) of the product was determined by potentiometric titration to be 0.43 and the molecular weight (MW) was established to be 277 kDa using high-performance liquid chromatography (HPLC) [23].

Methylene Blue, MB (Loba Feinchemie, Austria), was dissolved in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free, Oxoid Limited, UK) at a concentration of $3.125x10^{-2}$ mol/l and kept at room temperature. This stock solution was diluted shortly before use in PBS buffer to the final concentration of $3.125x10^{-5}$ mol/l and kept at 4 °C.

Isolation of the cells. Lymphocytes were isolated from blood taken by cardial punction followed by density centrifugation using Lymphocytes Separation Medium LSM 1077 (PAA Laboratories GmbH, Austria). Briefly, blood was taken from the heart using a heparinized hypodermic needle and carefully layered on separation medium (1:1). After centrifugation (2300 rpm, 25 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%.

Testicular cells were isolated from rat testes of sexually mature male SD rats by enzymatic digestion as described by BRADLEY and DYSART [24], with certain modifications suggested by SØNDERLUND et al [25]. 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%.

Hepatocytes were isolated by the *in situ* two step collagenase perfusion technique as described by MICHALOPOULOS et al [26]. First, the liver was perfused with a Ca^{2+}-free buffer solution not containing 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 greater than 80%.

Single cell gel electrophoresis. The procedure of SINGH et al [27] was used with minor modifications suggested by SLAMEŇOVÁ et al [28] and GÁBELOVÁ et al [29]. Briefly, $2x10^4$ 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. Treatment with visible light (60 W light bulb, 25 cm distance, 120 or 180 s) and Methylene Blue ($3.125x10^{-5}$ mol/l) was also carried out with cells embedded in agarose on microscope slides. The slides were then placed in lysing 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. For detection of oxidative DNA damage a modified comet assay according COLLINS et al [30] was used. After lysis, the gels were washed twice for 10 min with the endonuclease buffer (40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA, pH 8.0) and incubated with a cocktail of repair enzymes Fpg and EndoIII for 30 min at 37 °C. The control slides were incubated with endonuclease buffer containing

bovine serum albumin (BSA). After incubation, 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 value 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, Sigma Chemical Company, St. Louis, MO, 5 μg/ml).

EtBr stained nucleoids were evaluated with an Olympus BX-51 fluorescence microscope (Olympus Europa, Hamburg, Germany). 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, which is 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 treatment of visible light-excited Methylene Blue, 6 parallel slides (3 slides used for incubation with cocktail of enzymes and 3 slides were incubated without enzymes) have been prepared within one experiment. The significance of differences between samples was evaluated by Student's t-test (statistically decreased from untreated samples – "standard diet" – $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

Results

The connection between dietary intake of carboxymethyl chitin-glucan (CM-CG, approximately 200 mg/kg body weight, during 21 days) and the response of freshly isolated rat cells to genotoxic treatment with a combination of photosensitizer Methylene Blue and visible light (MB+VL) was evaluated in this study.

Figure 1 shows the percentage of DNA breaks after exposure to Methylene Blue and visible light (120 s and 180 s) detected in rat lymphocytes isolated from animals fed standard (left panel) or CM-CG supplemented diet (right panel). Methylene Blue in the absence of light (MB 180 s) caused only moderate formation of oxidative DNA lesions, in contrast to the case when visible light was also applied, where a pronounced increase of oxidative DNA modifications was detected ($^{***}p<0.001$). Lymphocytes isolated from animals fed CM-CG enriched diet were more resistant to genotoxic treatment than the lymphocytes from the control rats ($^{*}p<0.05$).

The protective effect of CM-CG containing diet against DNA lesions induced by MB+VL was also observed in testicular cells, as shown in Figure 2. Again, the cells isolated from CM-CG fed animals (right panel) were more resistant to the action of MB+VL. Interestingly, we detected an increased level of frank DNA breaks after MB+VL treatment in testicular cells from CM-CG fed animals compared to control ($^{*}p<0.05$, $^{***}p<.001$).

In hepatocytes (Fig. 3), the percentage of tail DNA determined by the modified comet assay was significantly increased after MB+VL treatment compared to control, while the levels of DNA damage in cells isolated from the rats fed the CM-CG supplemented diet (right panel) were lower ($^{***}p<0.001$) compared to the levels of DNA damage detected in hepatocytes of the control animals (left panel). In hepatocytes, the level of oxidative DNA damage did not reach values comparable with other cell types indicating their better protection against oxidative stress.

Discussion

Since clinical application of insoluble β-D-glucans is un-

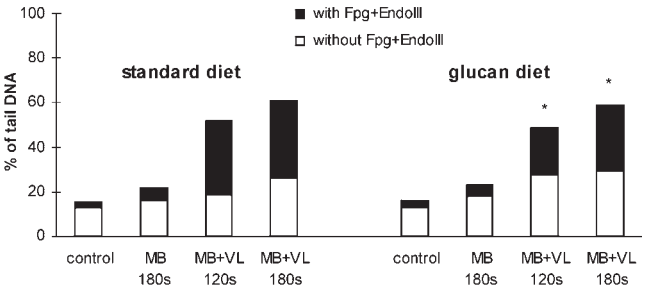

Figure 1. Levels of induced oxidative DNA damage in visible light-excited Methylene Blue (MB+VL) treated rat blood lymphocytes isolated from animals fed a standard diet (left panel) or carboxymethyl chitin-glucan (CM-CG) supplemented diet (right panel). The open parts of the bars represent DNA breaks (single strand DNA breaks and alkali labile sites), while the closed parts stand for additional Fpg+EndoIII sensitive sites as detected by a modified comet assay. Data represent means of four individual experiments (with three parallels each). Asterisks indicate significant decreases compared to rats fed with the standard diet ($^{*}p<0.05$).

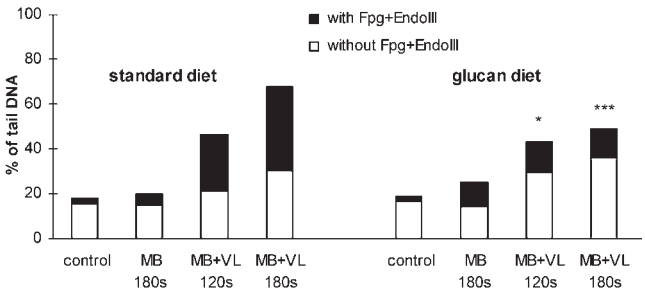

Figure 2. Levels of oxidative DNA lesions induced after treatment with MB+VL (120 s or 180 s) in testicular cells isolated from animals fed either a standard diet (left panel) or CM-CG enriched diet (right panel). The open portions of the bars correspond to DNA breaks (single strand DNA breaks and alkali labile sites), while the closed portions represent additional Fpg+EndoIII sensitive sites as detected by a modified comet assay. Data represent means of four individual experiments (with three parallels each). Asterisks indicate significant decreases compared to rats fed with the standard diet ($^{*}p<0.05$, $^{***}p<0.001$).

favorable due to their harmful side effects, synthesis of the water-soluble derivatives is required to develop a potentially applicable product. In order to modify the solubility and susceptibility to enzymatic degradation of insoluble β-D-glucans, several approaches have been used, such as ultrasonication, hydrolysis, or introduction of the charged groups (e.g., carboxymethyl, sulfoethyl, hydroxyethyl) into the molecule. Carboxymethyl chitin-glucan (CM-CG) used in this study is a water-soluble derivative that enables oral administration without causing adverse health effects such as hepatosplenomegaly, microembolization, and/or granulomatosis, which can arise upon parenteral application of the insoluble fungal β-D-glucans [9,10].

Although most of the studies performed with β-D-glucans concerned their immunomodulatory properties and the eventual therapeutic potential has been described, several investigations have been carried out to study their possible antimutagenic effect. Anti-clastogenic effect of β-D-glucans has been extensively studied by CHORVATOVIČOVÁ et al [12, 31, 32], CHORVATOVIČOVÁ and NAVAROVÁ [33], CHORVATOVIČOVÁ and ŠANDULA [11], CHORVATOVIČOVÁ [34] and TOHAMY et al [35]. PATCHEN et al [17], SAKAGAMI et al [18], BABINCOVÁ et al [19], KRIŽKOVÁ et al [14], SLAMEŇOVÁ et al [15], LAZAROVÁ et al [16], and KOGAN et al [20] have reported free radical scavenging activity for β-D-glucan or β-D-glucan-containing products. In contrast, TSIAPALI et al [22] described weak antioxidant activity of β-D-glucans and GÁBELOVÁ and PLEŠKOVÁ [20] also showed the failure of β-D-glucan derivative to inhibit oxidative DNA damage in human colonic cells Caco-2.

The aim of this study was to evaluate the possible protective effect of dietary intake of CM-CG against oxidative DNA damage induced by singlet oxygen ($^1O_2$) in freshly isolated rat cells. Blood lymphocytes, testicular cells, and hepatocytes were isolated from animals fed either a standard or CM-CG supplemented diet for 21 days (200 mg CM-CG/kg body weight). Highly reactive $^1O_2$ was generated during type-II photoreaction of photosensitization of Methylene Blue with visible light according to several authors who studied oxidative DNA damage [1, 36–40]. DNA lesions induced by $^1O_2$ are oxidative alterations to guanine residues (predominantly 7,8-dihydro-8-oxoguanine) which are recognized by site-specific DNA glycosylases (e.g., bacterial Fpg protein, mammalian Ogg1 protein) [41]. Detection of oxidative DNA lesions is enabled by a modified comet assay developed by COLLINS et al [30], which includes digestion with specific endonuclease (Fpg). Our results (Figs. 1–3) obtained by using a modified comet assay, demonstrate the ability of visible light-excited Methylene Blue to induce oxidative DNA damage in various types of freshly isolated rat cells. We also found out that in the cells isolated from animals supplemented with CM-CG enriched diet, the levels of oxidative DNA damage did not reach the values established in cells from the control animals. This is in accordance with our previous study, in which we have shown that the same dietary dose of CM-CG exhibits protective effect against DNA breaks induced by hydrogen peroxide in rat cells [16]. Earlier findings of KRIŽKOVÁ et al [14] and SLAMEŇOVÁ et al [15] demonstrated that CM-CG manifested its free radical scavenging activity and absorptive ability, which resulted in DNA protection. In addition, we studied the chemopreventive effects of CM-CG against the action of chemicals requiring metabolic activation (benzo[*a*]pyrene, dimethyldibenzocarbazole, *N*-nitrosomorpholine, and extractable organic matter from ambient air particles). Based on the observed inhibition of genotoxicity of the carcinogens, we assumed that the dietary supplementation of CM-CG might also affect the metabolism of xenobiotics by increasing the detoxification rate or by decreasing metabolic activation [42]. This assumption is supported by *in vivo* findings of TOHAMY et al [35], who demonstrated that β-D-glucans could decrease genotoxicity of the antineoplastic drugs cyclophosphamide, adriamycin, and cisplatin.

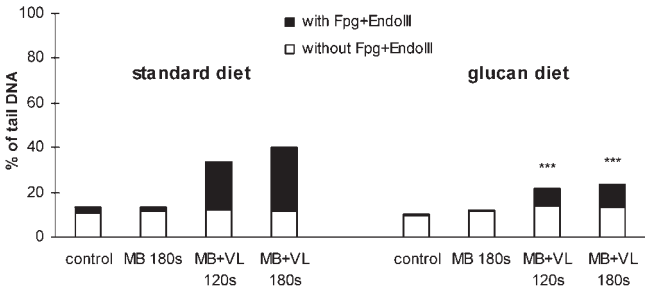

Figure 3. Levels of oxidative DNA damage induced by the photosensitization of MB with visible light (MB+VL) in hepatocytes isolated from rats fed a standard diet (left panel) or CM-CG supplemented diet (right panel). The open parts of the bars symbolize DNA breaks (single strand DNA breaks and alkali labile sites), while the closed parts represent additional Fpg+EndoIII sensitive sites as detected by a modified comet assay. Data represent means of four individual experiments (with three parallels each). Asterisks indicate significant decreases compared to rats fed with the standard diet (* $p<0.001$).**

Using different routes of application, CHORVATOVIČOVÁ and ŠANDULA [11] described beneficial effect of CM-CG against cyclophosphamide-induced DNA damage. They reported that antimutagenic effect of CM-CG was exerted upon intraperitoneal (i.p.), and intravenous (i.v.) administration, whereas oral (p.o.) application of CM-CG did not result in an anticlastogenic effect. This absence of chemopreventive effect of p.o. administration of CM-CG was explained by its insufficient absorption from the digestive tract due to its large size (600 kDa). On the other hand, ultrasonically fragmented CM-CG with lowered molecular weight (19 kDa) passed through the wall of the gastrointestinal tract and manifested its anticlastogenic effect [12]. In our study, a different design of treatment was used: we have compared the animals fed CM-CG enriched diet for 21 days (200 mg/kg body weight) with the control animals fed a standard diet. When added to the 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 oxidative stress induced by visible light-excited Methylene Blue. Molecular weight of CM-CG used in our experiments was 277 kDa, thus we assumed that CM-CG has effectively passed through gastrointestinal tract and manifested its protective effect as detected in different cell types. Recently, RICE et al [43] showed that soluble glucans (glucan phosphate, scleroglucan, and laminarin) could be translocated from gastrointestinal tract to the systemic circulation. The three soluble glucans were each absorbed after oral administration despite substantial differences in molecular weights, branching frequencies, and solution conformations.

In conclusion, we have demonstrated that carboxymethyl chitin-glucan represents a natural fungal polysaccharide that is able to exert antimutagenic properties upon application in diet. Nevertheless, additional data on the pharmacokinetics of CM-CG would be required to explain its fate in the animal organism.

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