

ANTICANCER ACTIVITY OF OAT β -GLUCAN IN COMBINATION WITH ELECTROPORATION ON HUMAN CANCER CELLS

ANNA CHOROMAŃSKA^{1*}, JULITA KULBACKA¹, JOANNA HARASYM²,
MAGDA DUBIŃSKA-MAGIERA³ and JOLANTA SACZKO¹

¹Department of Medical Biochemistry, Wrocław Medical University,
Chafubińskiego 10, 50-368 Wrocław, Poland

²Department of Food Biotechnology, Wrocław University of Economics,
Komandorska 118, 53-345 Wrocław, Poland

³Department of General Zoology, University of Wrocław, Sienkiewicza 21, 50-335 Wrocław, Poland

Abstract: The currently available data suggest that natural products may exert significant cytotoxic and immunomodulatory effects. Plant-derived chemotherapeutic agents such as taxol, etoposide or vincristine, currently used in cancer therapy, are prominent examples in this regard. However, there is a need for new and natural anticancer compounds with low or without toxicity to normal cells. One of the active compounds responsible for the immune effects is β -glucan derived from cereals, fungi, seaweeds, yeasts and bacteria. The recent data suggest that β -glucans are potent immunomodulators with anticancer properties. Antitumor properties of fungi and yeast derived β -glucans have been widely recognized, but those polysaccharides are mostly insoluble, creating several problems especially in topical formulation. To overcome the issue of low water solubility, in the current study a more soluble β -glucan type from oats was chosen for the investigation of its antitumor activities. Cytotoxic effects were studied using a human melanoma cell line (Me45). The effect of electroporation on the antitumor activity of oat β -glucan was investigated as well. Cellular viability assessment, immunocytochemistry and immunofluorescence were employed to evaluate biologic effects. Our results indicate strong anticancer properties of oat β -glucan, enhanced by electroporation.

Keywords: Oat β -glucan, electroporation, melanoma, apoptosis

Cancer, known medically as a malignant neoplasm, is a broad group of diseases involving unregulated cell growth. Through bloodstream or lymphatic system malignant tumors may invade nearby and distant parts of the body (1). Melanoma is the most dangerous form of skin cancer. Its advanced stages are very resistant to currently used treatments (1-3). Researchers are constantly looking for alternative methods against melanoma. Certain compounds isolated from plants show interesting therapeutic properties, including anticancer (4, 5). β -Glucans are natural polysaccharides of high medicinal and nutritional interest. They are found in the cell walls of yeasts, fungi, bacteria, algae, lichens, and plants, such as barley and oats (6). This polysaccharide is built from long, three-dimensional chains of β -glucose molecules, which is glycoside in position β (1-3), (1-4) or β (1-6), (7, 8). β -Glucans obtained from various sources have related biological

properties but they are different in structure, solubility in water and other solvents, and the particle size (9, 10).

Previous research indicated that β -glucans are natural products which have antitumor activity through a non-specific immune response (11, 12). These properties result from their degree of branching, conformation, structure, molecular weight and an isolation procedure (9). Early studies suggest that the mechanism of anticancer action is the result of activation of adaptive immune cells (CD4+ or/and CD8+ T cells) and B cells (11). The immune activation role of β -glucan in the anticancer properties is well documented (13, 14), however we think that this anticancer activity is more complex and complicated (6, 7, 15). The aim of our study was to evaluate the melanoma cytotoxicity and antitumor activities of β -glucan derived from oats *in vitro*. The antitumor properties were investigated by evaluation the

* Corresponding author: e-mail: anna.choromanska@umed.wroc.pl

expression of apoptotic proteins (cytochrome c, caspase 3 and caspase 12). Additionally, we tested the effects of electroporation-mediated transport enhancement of the oats β -glucan on cellular viability. The application of a high electric field to the plasma membrane generates transient hydrophilic electropores (16). The electroporation (EP) supports non-selective transport of non-permeant molecules into the cell and supported transport of drugs into cells. This procedure is known as electrochemotherapy (ECT) (17-19). Reversible electroporation can favor percolation of the drug into cells and increase the cytotoxic effect (1, 20). Electrochemotherapy has been widely used in the therapy of melanoma (18), but until now electroporation-mediated transport of β -glucan has not been studied.

EXPERIMENTAL

Cell culture

The human pigmented malignant melanoma (Me45) cell line (derived from a lymph node metastasis of skin melanoma in a 35-year-old male) was established in 1997 at the Radiobiology Department of the Center of Oncology in Gliwice, Poland. The cells were grown as a monolayer in Dulbecco modified Eagle medium (DMEM, Sigma-Aldrich, USA) which was enhanced with 2 mM L-glutamine, 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 50 μ g/mL streptomycin (Sigma-Aldrich, USA) at 37°C in 5% CO₂. Before the every experiments, the cells were removed by 0.25% trypsin with 0.02% EDTA (Sigma-Aldrich, USA).

Oats β -glucans recovery procedure

Oats β -glucan was obtained at the University of Economics in Wrocław following a procedure described elsewhere (21) with β -glucanase inactivation during lipid removal step, alkaline extraction, protein removal in isoelectric point, solution neutralization to pH = 7.0 and β -glucan precipitation

with ethanol. Low molecular oat 1-3, 1-4-D- β -glucan was manufactured following a procedure described elsewhere (22) with multistep freeze-milling of raw materials (20% β -glucan fiber, Microstructure, Poland), fat removal with ethanol extraction, alkaline extraction (pH 8-10) of β -glucan and oat proteins, protein precipitation and separation in isoelectric point, and finally, β -glucan precipitation with ethanol in equilibrium. β -Glucan purity was determined according to AOAC 995.16 method with test kit (Megazyme, Ireland) and was 84%. Molecular weight of oat β -glucan was measured with HPLC-SEC with guard column (OHpak SB-G, Shodex), a GPC column (SB-806M HQ, Shodex) and was 69650 g/mol. To prepare stock solution, two mg of oats β -glucan was dissolved in 1 mL of sterile distilled water and one drop of 10% NaOH was added. Then, the stock solution was incubated at 37°C for 24 h. The different concentrations of this compound were used to the studies (10, 20, 25, 50, 75, 100, 150, 200, 300, 400 and 500 μ g/mL).

Cellular viability - MTT assay

The viability of cells was determined by MTT assay (Sigma-Aldrich, USA) after experiments with different concentrations of β -glucan and after experiments where oat β -glucan transport was supported by electroporation. The MTT assay was used to estimate mitochondrial metabolic function through the measurement of mitochondrial dehydrogenase after 24 h incubation after experiments. For the experiment the cells were seeded into 96-well micoculture plates at 1×10^4 cells/well. After incubation with selected concentrations of β -glucan, the experiments were realized according to the manufacture's protocol. The absorbance was determined using a multi-well scanning spectrophotometer at 570 nm (EnSpire Perkin Elmer Multimode Plate Reader, USA). Mitochondrial metabolic function was expressed as a percentage of viable treated cells in relation to untreated control cells.

Table 1. The intensity of immunofluorescence reaction in Me45 cell line and the percentage of cells positively stained.

		The intensity of immunocytochemical staining	% of positively stained cells
Caspase 3	control cells	+	100
	cells + EP	+	100
	EP + 50 μ g/mL glucan	+++	100
	EP + 100 μ g/mL glucan	+++	100
	EP + 200 μ g/mL glucan	disintegration of cells	

Immunofluorescent assessment caspase 3 CLSM study

To prepare immunofluorescence reaction, the cells were grown on coverslips, then fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) in PBS, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, USA) in PBS (v/v) for 5 min and blocked with 1% FBS in PBS (for 30 min at room temperature). The cells were washed in PBS on the every step of procedure. The following antibodies were used: primary antibody monoclonal anti-caspase 3 antibody produced in mouse (overnight incubation at 4°C; 1 : 100; Santa Cruz Biotechnology, Inc., USA); secondary antibody goat anti-mouse IgG FITC conjugated (for 60 min. at room temperature; 1 : 50; Sigma-Aldrich, USA). DNA was stained with DAPI (4,6-diamidino-2-phenylindole, mounting medium Fluoroshield™, Sigma-Aldrich, USA). For imaging, Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Japan) was used. The images were recorded by employing a Plan-Apochromat 60× oil-immersion objective.

Immunocytochemical caspase 12 and cytochrome c evaluation

Immunocytochemistry was performed using the ABC method. The cultures were fixed and dehydrated using 4% PFA during 10 min. The samples were then permeabilized and blocked by incubation with 0.1% Triton X-100 in PBS. The enzymes expression were visualized with polyclonal antibody (1 : 100, anti-MnSOD, anti-cytochrome c; Santa Cruz, USA). For conventional bright-field microscopy (peroxidase-ABC labelling), the samples were

incubated with a diaminobenzidine-H₂O₂ mixture to visualize the peroxidase label and counterstained with hematoxylin for 30 s. The samples were analyzed with the upright microscope (Olympus BX51, Japan). Stained cells were determined by counting 100 cells in randomly selected fields. The result was judged positive if staining was observed in more than 5% of cells. The intensity of immunohistochemical staining was evaluated as: (-) negative, (+) weak, (++) moderate and (+++) strong.

Statistical analysis

Statistical significance was determined by unpaired Student's *t*-test vs. control untreated cells where $p < 0.05$ values were assumed as statistically significant. The results were analyzed statistically with Microsoft Office Excel 2010. All samples were analyzed in triplicate.

RESULTS

The results obtained from the MTT assay are presented in Figures 1 and 2. The studies showed that lower concentrations of oats β -glucan (10-150 μ g/mL) caused a slight decrease in cell viability (about 10%). The cytotoxicity of β -glucan increased starting from a concentration 200 μ g/mL. In this case the viability of cells reached 70%. At the highest tested glucan concentration it decreased to 19% (Fig. 1). The combination of oats β -glucan with electroporation caused stronger decrease in cells viability. However, at the concentration 200 μ g/mL it decreased to 12.5% (Fig. 2). Assessment of the level of expression of proteins involved in apoptotic pathways showed that oats β -glucan in concentra-

Table 2. The intensity of immunocytochemical reaction in Me45 cell line and the percentage of cells positively stained.

		The intensity of immunocytochemical staining	% of positively stained cells
Cytochrome c	control cells	-	0
	cells + EP	-	0
	EP + 50 μ g/mL glucan	+	40
	EP + 100 μ g/mL glucan	+	50
	EP + 200 μ g/mL glucan	disintegration of cells	
Caspase 12	control cells	-	0
	cells + EP	-	0
	EP + 50 μ g/mL glucan	-	0
	EP + 100 μ g/mL glucan	+	90
	EP + 200 μ g/mL glucan	disintegration of cells	

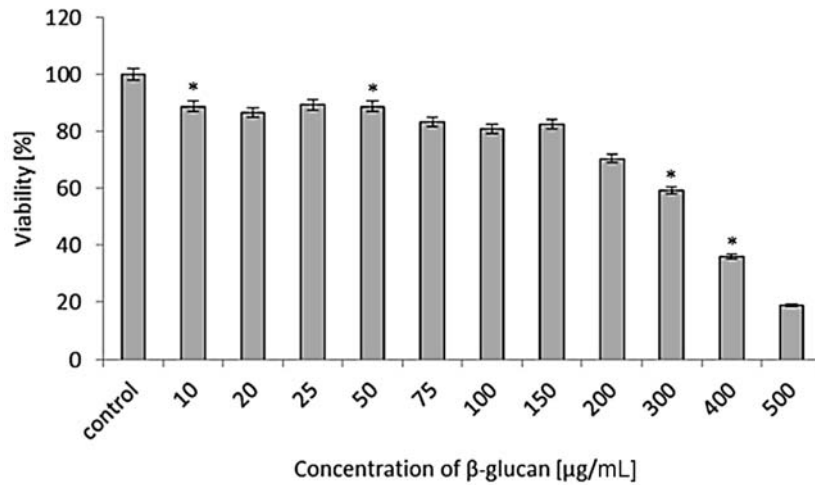


Figure 1. Viability of Me45 cell line after 24 h incubation following increasing concentrations of oat β -glucan. Viability is expressed as the percentage of the control cells (cells without oat β -glucan). Error bars shown are means \pm SD for $n = 3$. * statistically significant for $p \leq 0.05$.

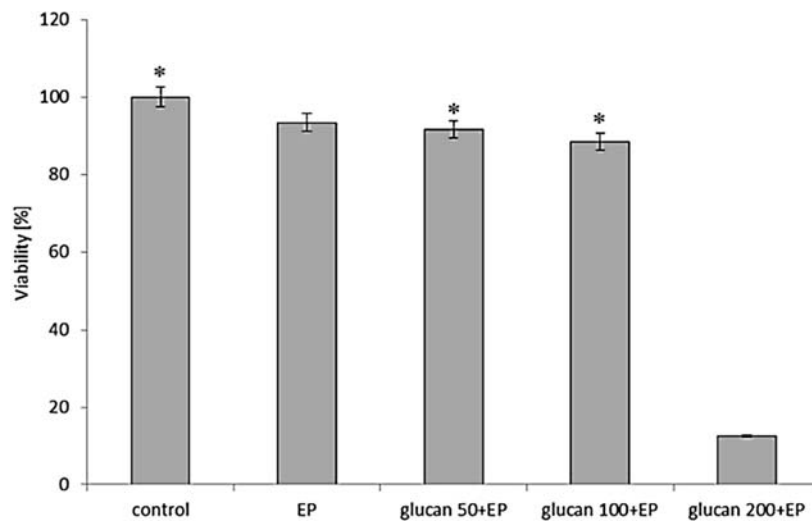


Figure 2. Viability of Me45 cell line after 24 h incubation following increasing concentrations of oat β -glucan in combination with electroporation. Viability is expressed as the percentage of the control cells (cells without oat β -glucan). Error bars shown are means \pm SD for $n = 3$. * statistically significant for $p \leq 0.05$.

tion 50, 100 and 200 $\mu\text{g/mL}$ in combination with EP significantly increased the level of caspase 3 expression, wherein the concentration 200 $\mu\text{g/mL}$ combined with EP causes cells degradation (Fig. 3, Table 1). Similar results were obtained for assessment of cytochrome c. A significant increase of cytochrome-c expression in cells incubated with β -glucan was observed and further enhanced by EP. β -Glucan in concentration 50 $\mu\text{g/mL}$ in combination with EP caused an increase of immunostained reac-

tion with cytochrome c released into the cytosol in 40% of observed cells. In concentration 100 $\mu\text{g/mL}$ and with EP it increased the number of positively stained cells to 50%. The highest tested concentration of glucan with EP resulted in destruction of the most evaluated cells (Fig. 4, Table 2). Immunocytochemical assay of caspase-12 showed a positive reaction in melanoma cells incubated with β -glucan at concentration 100 and 200 $\mu\text{g/mL}$ (Fig. 4 and Table 2).

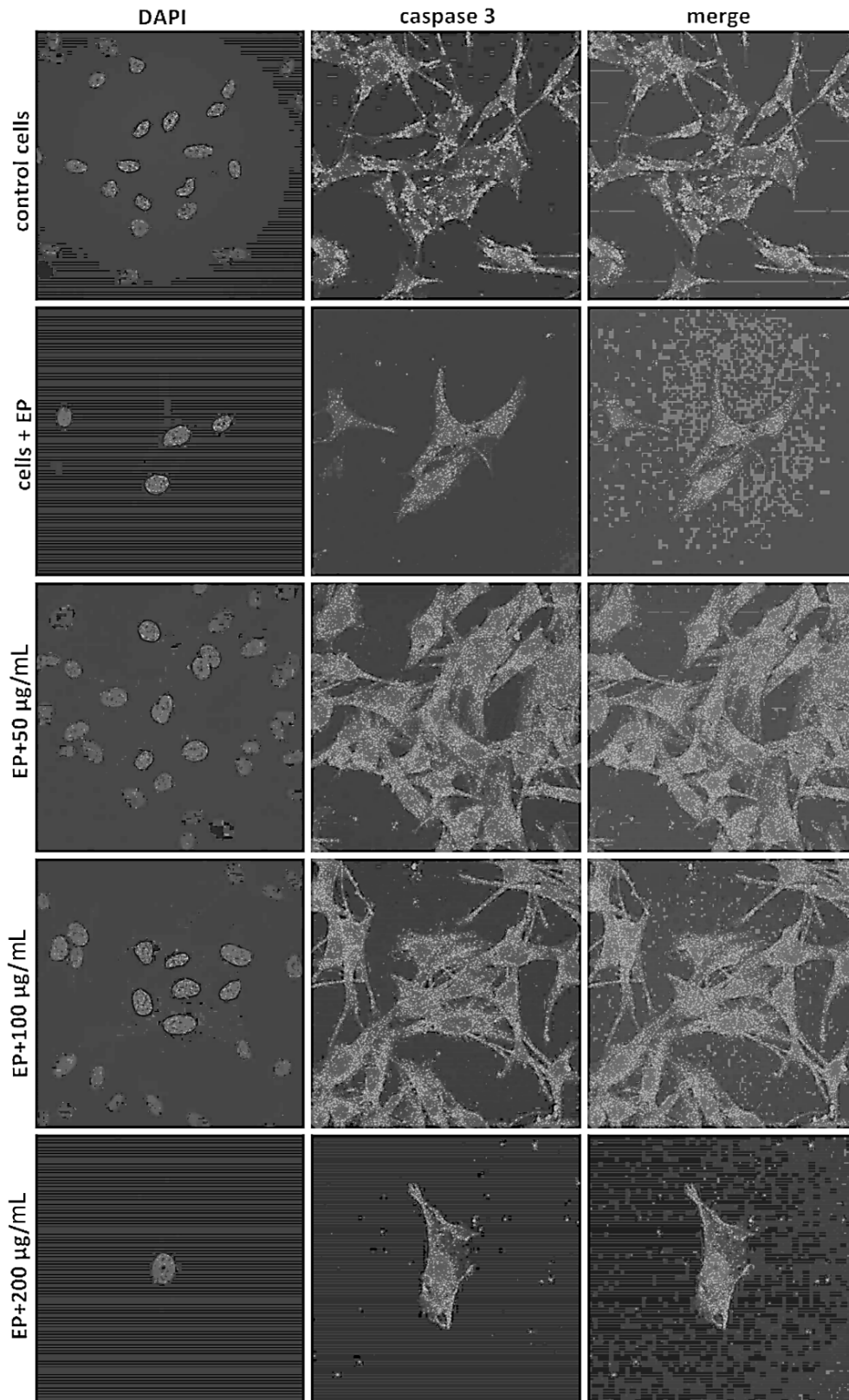


Figure 3. The level of caspase 3 expression in Me45 cell line after 24 h incubation following increasing concentrations of oat β -glucan in combination with electroporation

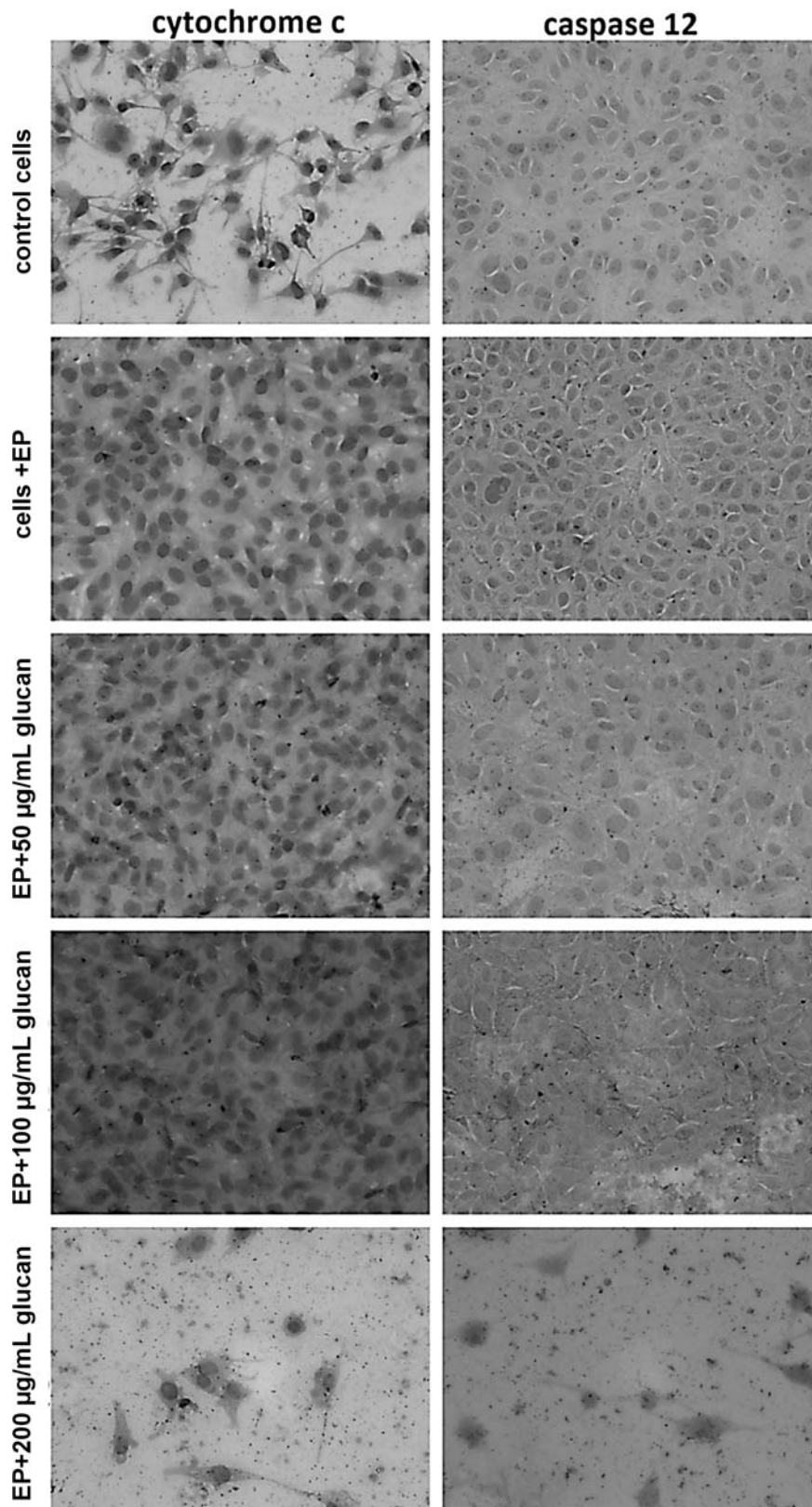


Figure 4. Cytochrome c and caspase 12 expression in Me45 cell line after 24 h incubation following increasing concentrations of oat β -glucan in combination with electroporation or negative control

DISCUSSION AND CONCLUSION

Various types of products containing β -glucans are used in cancer therapy combined with drugs in Asian countries. Previous investigations demonstrate that glucans have low cytotoxicity in normal cells and are well tolerated by patients (23). Although anticancer activities of glucans from mushrooms and bacteria are fairly well studied, there is still not enough information about anticancer properties of glucans derived from oats and so far its connection with electroporation has not been examined in human cancer cells. In our studies β -glucan induced cytotoxic effect at higher doses (above 200 $\mu\text{g/mL}$), but associating glucan with electroporation caused the highest decrease in cells viability to 12.5% at dose 200 $\mu\text{g/mL}$.

The results presented in this study regarding cellular viability after oats β -glucan incubation are consistent with previous reports of Parzonko et al. (6) and Choromańska et al. (7). However, a combination of electroporation with oat β -glucan caused the strongest cytotoxic effect. Importantly, as was shown in our previous studies and others (7, 8) that glucan does not cause toxic effects as compared to normal cells, representing a noteworthy phenomenon. In our study, it was also indicated proapoptotic potential of β -glucan in human melanoma cells. In contrast to necrosis, it doesn't induce any inflammatory reaction (24). During apoptotic pathway, mitochondria are induced to release cytochrome c into the cytoplasm. This mitochondrial pathway of caspase cascade activation is recruited in most forms of apoptosis. Previous observations showed that cancer cells are less susceptible to mitochondrial pathway of apoptosis because of higher mitochondrial membrane potential (25). The effective elimination of cancer cells may be based on successful disruption of the mitochondrial membrane potential (6). We observed marked significant increase of cytochrome c expression in cells treated with β -glucan and electroporation. In apoptosis, cytochrome c accumulates in the cytoplasm and binds to the Apaf-1. This complex activates the initiator caspase, procaspase 9 (26). Caspase 9 activates the caspase cascade, thus, cytochrome c acts as cofactor in the activation of the dormant killer proteases. Previous research of Parzonko et al. and Hirahara et al. showed a concentration dependent effect of β -glucan on the mitochondrial membrane potential. They also indicated initiation of apoptosis in tested cells and phosphatidylserine exposure on the external surface of the cells (6, 27). Some authors also indicated that endoplasmic reticulum (ER) stress can also cause apoptosis through caspase-12 activation (28). It has been reported to specif-

ically activate caspase-9, which can then cleave caspase-3 (29). In the present study, immunocytochemical analysis demonstrated an increase in the expression of caspase-12 and caspase-3 after oat β -glucan with electroporation. Similar results in *in vitro* study obtained Parzonko et al. and Xu et al. in *in vivo* experiments (6, 15).

There is still not specified mechanisms how β -glucan molecules are transported into cells. Supposedly, β -glucan toxicity against tumor cells may be related to the overexpression of the transporter responsible for the transport of glucose molecules in the cells. In this case, glucans - polymers composed of glucose units, are much more up-taken by tumor than normal cells. Increased GLUT1 (Glucose Transporter Type 1) expression has been demonstrated earlier in malignant melanomas (30). GLUT1 expression promotes glucose uptake and cell growth in that cells (31). Also in human melanoma tissues a significant correlation between GLUT1 expression and mitotic activity was found (30). Overexpression of GLUT1 protein confers poor prognosis in a wide range of solid tumors (32, 33).

We can conclude that oats β -glucan has a promising anti-melanoma potential which can be significantly enhanced by electroporation method. However, the mechanism of β -glucan action still requires further investigation.

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