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β-Glucan administration to diabetic rats reestablishes redox balance and stimulates cellular pro-survival mechanisms

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ABSTRACT

The mechanisms underlying the beneficial effect observed in diabetic rats after treatment with a commercially available β-glucan-enriched extract (BGEE) were examined. Multiple low-dose streptozotocin (STZ) diabetes was used (40 mg STZ/kg) as a model for type 1 diabetes. BGEE was administered daily (80 mg/kg) for 4 weeks, starting from the last day of the STZ treatment. *In vitro* and *in vivo* experiments suggest that significant free radical scavenging and antioxidant activities of BGEE were responsible for a systemic adjustment of the redox disturbance and reduction of DNA damage in the liver and kidney of diabetic rats. BGEE-treated diabetic rats also displayed increased Akt kinase activity and decreased pro-caspase-3 degradation, implying that BGEE mediates its beneficial effects through activation of the cellular pro-survival pathway. We conclude that β-glucan administration under diabetic conditions promotes a systemic improvement that can be expected to increase the organism's resistance to the onset of diabetic complications.

1. Introduction

Diabetes mellitus (DM) type 1 is a metabolic disorder characterized by hyperglycemia that results from a disruption of insulin-signaling because of insufficient insulin levels. The high glucose concentration in diabetes promotes the formation of noxious levels of free radicals through different routes: via glucose autoxidation (Martin, Salinas, Fujita, Tsuruo, & Cuadrado, 2002), as a result of the accumulation of advanced glycation endproduct (AGE) (Li, Sigmon, Babcock, & Ren, 2007), through the hexosamine biosynthesis pathway (Brownlee, 2001), due to the stimulation of the polyol pathway by unused glucose (Chung, Ho, Lam, & Chung, 2003), by protein kinase C activation (Noh & King, 2007), and as a result of mitochondrial overproduction of reactive oxygen species (ROS) (Nishikawa et al., 2000). While pancreatic oxidative

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Abbreviations: AGE, advanced glycation endproduct; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BGEE, β-glucan-enriched extract; D, diabetic animal group; ND, non-diabetic animal group; DM, diabetes mellitus; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MDA, malondialdehyde; NED, napthylethylenediamine dihydrochloride; SNP, sodium nitroprusside; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substance

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stress is an important contributing factor in the onset and progression of diabetes (Drews, Krippeit-Drews, & Düfer, 2010), the oxidative stress which accompanies the diabetic condition plays an important role in the development of diabetic pathologies (Giacco & Brownlee, 2010) that result from microvascular (diabetic retinopathy, nephropathy and neuropathy) and macrovascular complications (ischemic heart disease, cerebrovascular and peripheral vascular disease).

Diabetes management is based on synthetic agents and can also include a variety of nutritional supplements or nutriceuticals, commonly plant-derived active principles. Despite many strategies designed to improve different DM-related symptoms, treatment of diabetes remains far from satisfactory (Prabhakar & Doble, 2008). For one, diabetes management without any side-effects is still a challenge. Also, while therapeutic approaches in DM often target oxidative stress, antioxidant compounds in the form of vitamins A, C and E have yielded somewhat limited results (Wiernsperger, 2003). It is therefore imperative to continuously identify, select and process novel natural products with potentially fewer side effects for use in "causal" therapy of diabetes management (Cariello, 2003) based on antioxidant protection (Robertson, 2010).

Among the different naturally-occurring substances, it has been suggested that cereals are a useful source of active principles with free radical scavenging activities (Djordjevic, Šiler-Marinkovic, & Dimitrijevic-Brankovic, 2011). Also, some of the biological activities of edible mushrooms are due to the presence of dietary fiber, chitin in particular, and notably the β-glucans (Mattila et al., 2001). Natural products containing β-glucans have been used for centuries in human nutrition. The β-glucans are a diverse group of polysaccharides of D-glucose monomers linked by β-glycosidic bonds. Most commonly, they occur as cellulose in plants, in the bran of cereal grains and as principal components of cell walls of baker's yeast, certain mushrooms and fungi (Roupas, Keogh, Noakes, Margetts, & Taylor, 2012; Zekovic, Kwiatkowski, Vrvic, Jakovljevic, & Moran, 2005). The β-glucans possess a variety of biological activities. They are noted for their ability to modulate the immune system and for their anti-infective and anti-tumorigenic properties (Brown & Gordon, 2003). Also, dietary intake of β-glucans has been shown to reduce risk factors and to benefit the treatment of DM and associated complications by virtue of their ability to lower hyperglycemia, hyperlipidemia and hypertension (Kuda, Toshiki Enomoto, & Yano, 2009; Rahar, Swami, Nagpal, Nagpal, & Singh, 2011).

The aim of the present study was to examine the effects of the administration of a β-glucan-enriched extract (BGEE) from cereal grain and mushroom on the redox status of STZ-induced diabetic rats. We wanted to determine whether BGEE causes a significant enough attenuation of diabetes-associated oxidative stress to suppress the pro-apoptotic pathway which assumes a critical role in the pathogenic mechanisms in diabetes. We observed that the continuous administration of BGEE to diabetic rats during the first 4 weeks post induction of experimental diabetes caused a substantial reduction of hyperglycemia and of triglyceride, cholesterol and creatinine levels. The observed systemic improvement was accompanied by decreased lipid peroxidation, restoration of antioxidant enzyme activities and by Akt kinase activation. These results point to a potentially important beneficial role of the examined β-glucan-enriched preparation in diabetes management.

2. Experimental part

2.1. Materials

The β-glucan-enriched extract (BGEE) from cereal grains is for human consumption and is produced by Essentia s.r.o. in the Slovak Republic. The BGEE examined herein was supplied by Agrobiv d.o.o. (Croatia). The BGEE is a concentrate of colloid micronized β-glucan (⩾80%). Based on methylation analysis, the material was characterized as a 1,3 β-D-glucan with a small number of 1,6 and 1,4 linked glucose residues. The maximal protein, fat and sodium contents in the standardized dry substance are 20, 10 and 15 mg/g, respectively. Routine microbiological testing did not reveal the presence of any contaminants. The quality of the BGEE is covered by the Essentia certificate. For the extraction procedure and quantitative measurement of the β-glucans refer to Karácsonyi and Kuniak (1994) and Hozová, Kuniak, Moravčíková, and Gajdošová (2007) who examined 43 samples of six cereal varieties (oats, barley, wheat, millet) and pseudocereal (buckwheat, amaranth). Streptozotocin (STZ) was obtained from MP Biomedicals (Solon, OH, USA).

2.2. Animals

Experiments were performed on 2.5-month-old adult male albino Wistar rats weighing 220–250 g. All the animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health.

2.3. Experimental protocol

The experimental model of type 1 DM in male albino Wistar rats was induced by intraperitoneal (i.p.) injections of STZ (40 mg/kg/day) for five consecutive days. STZ was dissolved immediately before use in sodium citrate buffer (0.1 M, pH 4.5). The blood glucose level was measured 24 h after the last STZ injection. Diabetes was defined by a fasting blood glucose level that exceeded 20 mmol/l. Blood samples were obtained from the tail vein from rats that fasted overnight. Glucose was measured with a blood glucosemeter (Accu-Chech Active). In our hands, the described treatment with multiple low doses of STZ did not cause animal death. The rats were randomly divided into four groups; these are: (i) a negative control or the non-diabetic group (ND) which received an equivalent volume of citrate buffer (i.p.) for five consecutive days ($n = 7$); (ii) a BGEE-treated non-diabetic group or the positive control which received a daily dose of 80 mg BGEE suspended in water/kg (i.p.) for 4 weeks ($n = 7$); (iii) a diabetic group (D) which received STZ as described above; these rats were left untreated throughout the 4 week period ($n = 8$); (iv) a diabetic group treated with BGEE ($n = 8$) which received a

daily dose of BGEE as described above, lasting 4 weeks, starting from the last day of STZ administration.

2.4. DPPH radical-scavenging assay

The *in vitro* free radical-scavenging activity of BGEE was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl), which is a stable free radical with a maximal absorbance at 517 nm. One milliliter of a 0.3 mM DPPH ethanolic solution was added to 2.5 ml of sample solutions of different concentrations (20–100 mg/ml) and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm. Ascorbic acid was used as a standard. All tests were performed in triplicate. The inhibitory percentage of DPPH was calculated according to the formula: Inhibition = $[(A_{blanc} - A_{test})/A_{blanc}] \times 100$. A_{blanc} is the absorbance of the DPPH in solution without the test sample and A_{test} is the absorbance of DPPH in the solution with the test sample.

2.5. Fe^{2+} chelation

To determine the chelating activity of ferrous ions, different concentrations of BGEE (20–200 mg/ml) were dissolved in 0.25 ml of 0.125 mM $FeSO_4$. The reaction was initiated by the addition of 0.3125 mM ferrozine (0.25 ml). The mixture was shaken vigorously and left standing at room temperature for 10 min. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation (IFCF) was calculated using the formula: $IFCF = [(A_{control} - A_{sample})/A_{control}] \times 100$.

2.6. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability of the BGEE was determined according to the following procedure. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of extracts (20–200 mg/ml) in 250 μl distilled water were added to a H_2O_2 solution (40 mM in 600 μl). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of H_2O_2 scavenging of extracts and standard compounds (ascorbic acid) were calculated from the formula: H_2O_2 scavenging effect = $[(A_{control} - A_{sample})/A_{control}] \times 100$. $A_{control}$ is the absorbance of the phosphate buffer with H_2O_2 and A_{sample} is the absorbance in the presence of the extracts or standards.

2.7. Nitric oxide radical scavenging

The nitric oxide generated from aqueous sodium nitroprusside (SNP) solution at physiological pH interacts with oxygen to produce nitrite ions, which can be quantified by the Griess-Illosvoy reaction. The reaction mixture contained 10 mM SNP in phosphate buffered saline (pH 7.4) and different concentrations of the BGEE (20–200 mg/ml) in a final 3 ml volume. After incubation for 150 min at 25 °C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 500 μl of the incubated solution and allowed to stand for 5 min. One milliliter of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was then added and the mixture was incubated for 30 min at 25 °C. The generated pink chromophore was measured spectrophotometrically at 540 nm. The percentage of nitric oxide (NO) scavenging of extracts and standard compounds (curcumin) was calculated from the formula: NO scavenging effect = $[(A_{control} - A_{sample})/A_{control}] \times 100$. $A_{control}$ is the absorbance of the reaction mixture without extracts or standard and A_{sample} is the absorbance of the reaction mixture in the presence of the extracts or standards.

2.8. Preparation of serum

Serum was obtained after blood clotting and centrifugation at 2000*g* for 10 min. Serum was used for the determination of blood glucose, triacylglycerol, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN), creatinine levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinaze (CK) activities.

2.9. Determination of general biochemical parameters

Blood glucose levels in all four experimental groups were recorded at the beginning of the experiments, at 24 h after administration of the last dose of STZ and 4 weeks after the treatment, using a commercial kit (Gluco-quant Glucose/HK; Boehringer Mannheim, Mannheim, Germany) which is based on the hexokinase/G6P-DH enzymatic method. Hemoglobin (Hb) was determined according to Drabkin and Austin (1935). Glycosylated hemoglobin (GlyHb) was determined by the colorimetric assay according to Parker, England, DaCosta, Hess, and Goldstein (1981). Serum triacylglycerol were measured by the GPO-PAP method with an enzymatic kit (Randox Laboratories, Crumlin, UK). The total cholesterol level was measured by the enzymatic cholesterol esterase-cholesterol oxidase method using the CHOD-PAP test kit (Randox Laboratories, Crumlin, UK). HDL-cholesterol was measured by HDL-cholesterol liquid assay and LDL-cholesterol was calculated according to Friedewalds formula: LDL (mmol/l) = total cholesterol − (triacylglycerol/2.2) − HDL-cholesterol. Activities of ALT and AST were estimated in the serum by measuring the produced oxaloacetate and pyruvate, respectively, using an optimized standard UV kinetic method kit (GPT (ALAT) IFCC mod.; GOT (ASAT) IFCC mod). Plasma creatinine concentrations were determined using Cayman's Creatinine (serum) Assay with the Jaffe rate method according to the manufacturer's instructions. Blood urea nitrogen (BUN) level was estimated according to GLDH method (Human, Wiesbaden, Germany). Creatine kinase (CK) activity was evaluated according to CK NAC method, liquid NAC activate UV test (Human, Wiesbaden, Germany). The serum iron content was evaluated using the Ferrimat-Kit (bioMerieux SA, Marcy-l'Etoile, France). The ferrous iron forms a colored complex with ferrozine. The intensity of the color at 590 nm is directly proportional to the iron concentration in the sample. In order to determine the concentration of free ferrous iron (vs. total iron) in the serum, we conducted the same procedure as for total iron determination, but excluded the reductant hydroxylamine from the assay procedure.

2.10. Preparation of liver and kidney homogenates

Livers and kidneys from all experimental groups were excised and homogenized in 0.25 M sucrose, 0.1 M EDTA and 0.05 M Tris–HCl, pH 7.4. After sonication, the homogenate was centrifuged at 100,000*g* in a Beckmann Ti 50 rotor for 90 min. Aliquots of the supernatant were stored at −80 °C and used for the TBARS assay and determination of catalase (CAT) and superoxide dismutase (SOD) activities.

2.11. Thiobarbituric acid-reactive substance (TBARS) assay

The basic principle of the TBARS method is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at 95 °C. Briefly, an aliquot of the homogenates (0.1 ml) was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% TBA and 0.7 ml water and heated at 95 °C for 60 min. After cooling to room temperature, 1 ml of water and 5 ml of *n*-butanol/pyridine (15:1, v/v) were added, mixed and centrifuged at 3000*g* for 10 min. The red pigment in the supernatant fractions was estimated by absorbance at 532 nm. A calibration curve was prepared with 1,1,3,3-tetramethoxypropan (concentrations ranged from 25 nmol/ml to 1 μmol/ml).

2.12. Determination of SOD and CAT activities

Liver and kidney homogenates were used for the determination of CAT activity by the rate of hydrogen peroxide decomposition, expressed as μmol H_2O_2/min/g wet mass. Total SOD activity was measured by the epinephrine method, which is based on the capacity of SOD to inhibit the auto oxidation of epinephrine to adrenochrome, and was expressed as U/g wet mass. Mn-SOD activity was performed after preincubation with 8 mmol/l KCN; CuZnSOD activity was calculated from the difference between total SOD and MnSOD activities.

2.13. Comet assay

DNA fragmentation was detected using the alkaline comet assay. Liver and kidney samples from all experimental groups were minced in ice-cold HBSS buffer (0.14 g/l $CaCl_2$, 0.4 g/l KCl, 0.06 g/l KH_2PO_4, 0.1 g/l $MgCl_2 \times 6H_2O$, 0.1 g/l $MgSO_4 \times 7H_2O$, 8.0 g/l NaCl, 0.35 g/l $NaHCO_3$, 0.09 g/l $Na_2HPO_4 \times 7H_2O$, 1.0 g/l D-glucose) containing 20 mM EDTA and 10% DMSO. The solution was aspirated and a fresh solution was added to the cell mass. The procedure was repeated three times and the final cell suspension was collected. Ten microliter of cell suspension were mixed with low-melting agarose and applied to a microscope slide. Cells were lysed for 2 h at 4 °C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100). After lysis, the slides were incubated for 30 min at 4 °C in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) and subjected to electrophoresis in order to separate the damaged DNA fragments. The slides were placed in neutralization buffer (0.4 M Tris–HCl, pH 7.4) and stained with Syber Green I (Sigma–Aldrich, St. Louis, MO, USA). DNA damage was quantified by measuring the displacement between the genetic material contained inside the nuclear sphere (comet 'head') and the resulting comet 'tail'. The 'tail moment' has been suggested to be an appropriate index of induced DNA damage (considering both the migration of the genetic material and the relative amount of DNA in the tail. Images were analyzed with TriTekCometScore™ Freeware v1.5, which is available at: http://www.AutoComet.com.

2.14. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis

Twenty micrograms of liver and kidney homogenates were loaded onto 4% stacking/12% separating slab gels. After electrophoresis, proteins were transferred to PVDF membranes (Amersham Hybond-P, GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire, UK). Immunoblot analysis was performed using polyclonal antibodies to Akt 1/2/3, phosphorylated Akt 1/2/3 (Ser 473) (pAkt), caspase-3, tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with blocking solution (0.05% Tween 20, 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 3% non-fat condensed milk), the membranes were incubated with antibody for 2 h at room temperature. After rinsing, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin for 1 h. Immunoreactive bands were identified by an enhanced chemiluminescence detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions.

2.15. Statistical analysis

The data were expressed as the mean ± S.E.M. (standard error of mean). Statistical differences between groups were analyzed using one-way Analysis of Variance (ANOVA), followed by Duncan's multiple range test. The difference was considered statistically significant at $p < 0.05$.

3. Results

3.1. In vitro antioxidant and free radical scavenging activity of BGEE

Determination of antioxidant activities is widely used to characterize foods and preparations obtained from medicinal plants and fungi. In this study, different aspects of the antioxidant activity of BGEE were evaluated using the following *in vitro* tests: DPPH radical scavenging activity (Fig. 1a), ferrous ions chelating capacity (Fig. 1b), hydrogen peroxide scavenging activity (Fig. 1c) and nitric oxide radical scavenging capacity (Fig. 1c). DPPH has been widely used to evaluate the free radical scavenging effectiveness of antioxidant substances. Increasing scavenging activities (i.e. decreasing concentrations of the DPPH radical) were observed at increasing concentrations of BGEE (Fig. 1a). Chelating agents can function as secondary antioxidants which reduce the redox potential by stabilizing the oxidized form of metal ions. The ferrous ion chelating activity of BGEE is shown in Fig. 1b. BGEE exhibited a significant concentration-dependent metal chelating activity. The hydrogen peroxide that is formed *in vivo* as a result of the activity of oxidases mediates oxidative DNA damage. As can be seen on Fig. 1c, BGEE exhibited a

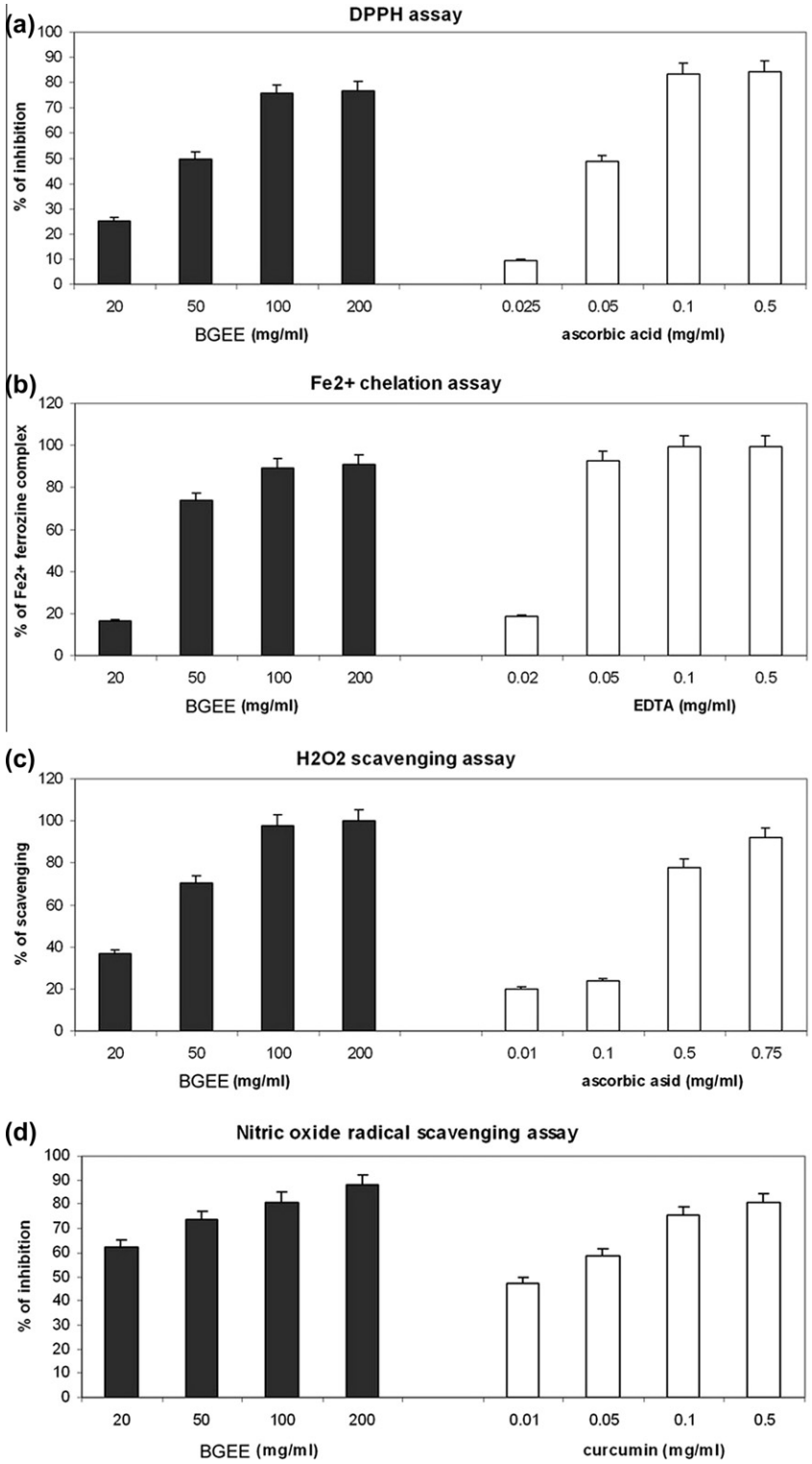

Fig. 1 – *In vitro* antioxidant and scavenging activity of BGEE. (a) DPPH radical scavenging activity; (b) ferrous ions chelating capacity; (c) hydrogen peroxide scavenging activity; (d) nitric oxide radical scavenging capacity of different concentrations of BGEE. Ascorbic acid, EDTA and curcumin were used as standards. The results of the assays are presented as the means ± S.E.M. from six separate measurements.

concentration-dependent hydrogen peroxide scavenging effect. Nitric oxide (NO) is an important cellular signaling molecule and low levels of NO production participate in many physiological processes. However, chronic production of NO is toxic to tissues and is associated with various pathological processes, including inflammatory conditions in juvenile diabetes. BGEE displayed a significant, dose-dependent ability to scavenge the NO radical *in vitro* (Fig. 1d).

3.2. Changes in biochemical parameters of diabetes

The biochemical markers of diabetes in different experimental groups after the indicated treatments are presented in Table 1. The treatment with STZ induced diabetes, observed as a more than 7-fold increase in blood glucose concentration. While the treatment of diabetic rats (D) with BGEE lowered the glucose concentration, the concentration of glucose remained 3.4-fold higher than in the untreated non-diabetic rats (ND). Under diabetic conditions, the level of GlyHb was 2-fold higher while the BGEE treatment decreased it to 1.2-fold above the control level. The diabetic rats presented 1.5-, 3.9- and 2.9-fold increases in serum creatinine, blood urea nitrogen (BUN) concentrations and CK, respectively, in comparison to the control group. Higher AST and ALT activities (2- and 3.4-fold, respectively), compared to the untreated control group were also measured. BGEE administration to diabetic rats promoted a decrease in creatinine, BUN and CK concentration, albeit to levels that were 1.3-, 2.5- and 2-fold above the normal concentration, and lower AST and ALT activities (1.6- and 3.1-fold above the control value, respectively). The treatment of control ND rats with BGEE did not cause significant changes of any of these markers. In contrast, treatment of ND control rats with BGEE led to lower concentrations of triacylglycerols and cholesterol (2- and 1.2-fold, respectively). While the concentrations of triacylglycerols and cholesterol were respectively 1.3- and 1.35-fold above the normal levels in diabetic rats, the treatment of diabetic rats with BGEE lowered the triacylglycerol and cholesterol levels 2.7- and 2.3-fold below the respective control levels. HDL concentrations did not statistically differ between the experimental groups, while LDL was 1.7-fold higher in the diabetic group compared to the control. The BGEE treatment reduced LDL 4-fold compared to the intact control. The levels of serum iron did not display any significant differences between the experimental groups, however, serum Fe^{2+} was increased 3.4-fold in the diabetic group; after administration of BGEE while the concentration of serum Fe^{2+} was lower, it was still 2-fold higher than in the control ND group.

3.3. Assessment of changes in lipid peroxidation as an indicator of oxidative stress in liver and kidney

Lipid peroxidation is one of the harmful consequences of the formation of reactive species and is a widely used biomarker of oxidative stress. Lipid peroxidation is a reflection of irreversible oxidative changes, including damage to different cellular membranes and impaired organelle functions that lead to cell dysfunction. Using the TBARS method, the redox status in the liver and kidneys was distinguished by different levels of lipid peroxidation in each experimental group (Fig. 2). The level of TBARS as a typical byproduct of lipid peroxidation served as an indicator of lipid peroxidation in liver and kidneys. The results presented in Fig. 2A show that TBARS were 1.3-fold increased in the livers of diabetic rats and that the treatment with BGEE lowered lipid peroxidation below the control level. Treatment of control ND rats with BGEE did not significantly change the TBARS levels. A similar trend was observed in the kidneys (Fig. 2B). In diabetic rats, TBARS were increased 1.6-fold above the control level. The treatment of diabetic rats with BGEE lowered TBARS below the control value.

3.4. Antioxidative enzyme activities in the liver and kidneys

Disturbances of antioxidant defenses in diabetes involve alterations in activities of antioxidant enzymes. The activities of the major antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) provide the first line of defense against increased ROS levels. The activities of total SOD, CuZnSOD and MnSOD were decreased 1.4-fold in the livers

Table 1 – Biochemical parameters in untreated and BGEE-treated non-diabetic and diabetic rats at 4 weeks after administration of the last dose of STZ.

	Experimental groups			
	ND		D	
	Control	BGEE	Control	BGEE
N (number of rats)	7	7	8	8
Glucose (mmol/l)	5.4 ± 0.2^{a}	5.1 ± 0.2^{a}	39.1 ± 105^{b}	18.6 ± 0.9^{c}
GlyHb (μmol/g Hb)	9.8 ± 0.4^{a}	7.9 ± 0.3^{a}	18.5 ± 0.8^{b}	11.5 ± 0.4^{c}
Triacylglycerols (mmol/l)	0.97 ± 0.04^{a}	0.51 ± 0.02^{b}	1.31 ± 0.06^{c}	0.35 ± 0.01^{d}
Cholesterol	2.07 ± 0.1^{a}	1.7 ± 0.08^{b}	2.8 ± 0.1^{c}	0.9 ± 0.03^{d}
HDL (mmol/l)	0.6 ± 0.02^{a}	0.6 ± 0.01^{a}	0.5 ± 0.02^{a}	0.55 ± 0.01^{a}
LDH (mmol/l)	1.03 ± 0.04^{a}	0.87 ± 0.03^{a}	1.71 ± 0.07^{b}	0.25 ± 0.01^{c}
AST(U/l)	174 ± 6.7^{a}	158 ± 6.3^{a}	357 ± 14.1^{b}	277 ± 11.1^{c}
ALT (U/l)	56 ± 2.2^{a}	43 ± 1.7^{a}	193 ± 7.7^{b}	174 ± 6.8^{c}
Creatinine (μmol/l)	29 ± 1.1^{a}	29 ± 1.2^{a}	43 ± 1.8^{b}	38 ± 1.5^{c}
Creatine kinaze (U/l)	21.4 ± 0.8^{a}	20.8 ± 0.8^{a}	60.6 ± 2.4^{b}	46.7 ± 1.8^{c}
BUN (mmol/l)	5.5 ± 0.2^{a}	4.6 ± 0.2^{a}	21.8 ± 0.9^{b}	14.2 ± 0.6^{c}
Serum Fe (μg/dl)	75.36 ± 3.8^{a}	73.55 ± 3.7^{a}	85.93 ± 5.1^{a}	79.95 ± 4.2^{a}
Serum Fe2+(μg/dl)	9.4 ± 0.3^{a}	9.6 ± 0.3^{a}	31.6 ± 1.3^{b}	18.4 ± 0.7^{c}

ND – non diabetic animals. D – diabetic animals. BCEE – β-glucan enriched extract.
Values are means ± S.E.M for the indicated number of rats in each group (*N*).
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

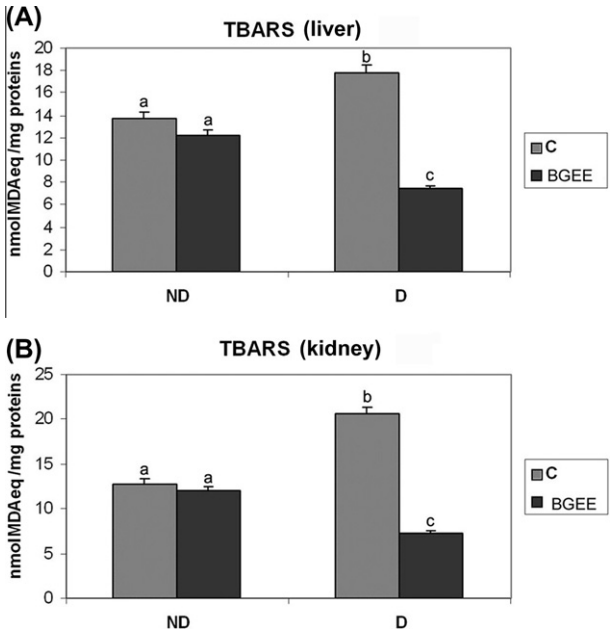

Fig. 2 – The effect of BGEE on lipid peroxidation (TBARS) in the liver (A) and kidneys (B) of non-diabetic and diabetic rats. The values are presented as the mean ± S.E.M. for the indicated number of animals (N) in each group (see Table 1). ND – non-diabetic group; D – diabetic group. Means that do not share a common letter are significantly different between groups ($p < 0.05$).

of diabetic animals (Fig. 3A). While the treatment with BGEE brought about an increase in their activities, they remained below the levels measured in control rats. The activity of CAT (Fig. 3B) in the liver of the diabetic group exhibited a sharp decrease (1.9-fold); in diabetic rats that were treated with BGEE while CAT activity increased, it still remained 1.5-fold lower than in the control. BGEE-treated ND rats did not exhibit statistically significant changes in SOD and CAT activities in comparison with the controls. Total SOD, CuZnSOD and MnSOD activities in the kidney (Fig. 3C) underwent similar changes. Under diabetic conditions the activities of both total SOD and CuZnSOD decreased 1.2-fold and of MnSOD 1.4-fold. The treatment with BGEE recovered the enzymatic activities fully to the control level. CAT activity in the kidneys was about 2-fold lower in diabetic rats (Fig. 3D). The treatment with BGEE increased CAT activity, albeit to a 1.5-fold lower level than in the control. BGEE-treated non-diabetic rats did not exhibit any differences in the examined antioxidant enzyme activities when compared to the intact control.

3.5. *Estimation of DNA damage levels in the liver and kidneys*

Besides lipids and proteins, DNA is a very important target in oxidative stress-promoted damage. The levels of primary DNA damage in the liver and kidney were assessed by the comet assay which examines the displacement of fluorescence from the nucleus, i.e. the comet head, to its tail which is observed only in cells whose DNA was damaged. The tail moment represents the relative index of the induced DNA damage. As can be seen on Fig. 4a, a 46-fold increase in DNA damage was observed in the liver of diabetic rats. The treatment with BGEE significantly reduced the level of DNA damage to a level that was 9-fold higher than in the intact ND control. The level of DNA damage in the kidney of diabetic rats was 13-fold higher than in the control group. The treatment with BGEE lowered DNA damage to a level that was 6.5-fold higher than in the controls. Since the tail moment in the liver and kidneys of the BGEE-treated ND rats did not statistically differ from that in the ND control group, we concluded that the extract did not exert any apparent genotoxic effects in either the liver or the kidneys.

3.6. *Relative changes in Akt kinase activity and pro-caspase-3 degradation in the liver and kidneys*

Oxidative stress stimulates opposing pathways that signal cell survival and cell death (Martin et al. (2002)) and a complex cross-talk between these pathways ultimately resolves cell fate. We investigated the best-characterized molecular players in these opposing signaling pathways: the pro-survival Akt kinase and the pro-apoptotic executioner caspase-3. Akt kinase, as the principal mediator of cellular pro-survival signals, can serve as an indicator of cell viability. Immunoblot analysis revealed that Akt activity was slightly lower in diabetic rats. This was observed as a decrease in the level of phosphorylated Akt kinase (pAkt) in both the liver and kidneys (Fig. 5, lane 2; row pAkt). The treatment with BGEE completely restored the level of active phosphorylated Akt kinase in the liver to the control level. While Akt activity in the kidney also increased, it remained slightly below the control level (lane 3; row pAkt). These findings are also presented on the graph in Fig. 5 in which the pAkt/Akt ratio is shown. The caspases are the major effectors of cell death. Using immunoblot analysis, we examined pro-caspase-3 degradation, the essential step that triggers the release of active caspase-3. As shown in Fig. 5, the treatment with BGEE prevented pro-casapse-3 degradation in the liver and in the kidneys (Fig. 5, lane 3; row pro-caspase). Caspase-3 activation was observed in both the liver and kidneys of diabetic rats (Fig. 5, lane 2; row pro-caspase and the graph below the immunoblot analysis). These results clearly reveal an important cytoprotective effect of administered BGEE.

4. Discussion

The results presented in this study show that the daily administration of BGEE exhibits a beneficial effect at different levels on several pathogenic events that accompany diabetes. The observed sum of improvements of the major biochemical indicators of diabetes (Table 1) led us to conclude that BGEE administration exerted a beneficial systemic effect by alleviating the upstream and downstream events which contribute towards increased oxidant stress in diabetes. BGEE administration lowered diabetic hyperglycemia and alleviated dyslipidemia by decreasing the triglyceride and cholesterol levels. As a result of its ability to adjust the diabetes-associated redox disturbance and quench stress signals at different points, BGEE significantly lowered DNA damage. It thereby demon-

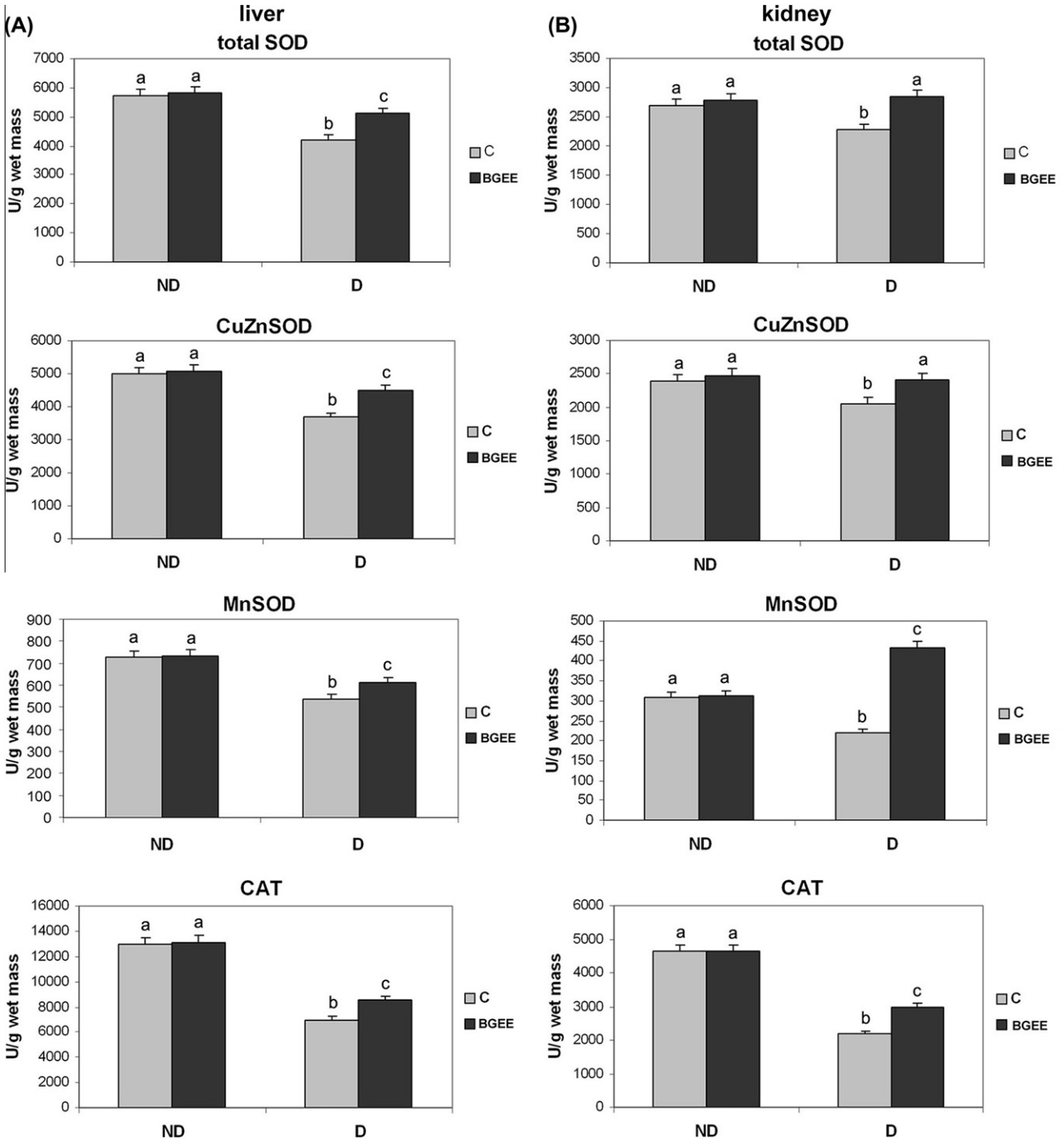

Fig. 3 – The effect of the treatment with BGEE on the activities of total SOD, CuZnSOD, MnSOD and CAT in the liver (A) and kidneys (B) of non-diabetic and diabetic rats. Values are the mean ± S.E.M. for the indicated number of animals (*N*) for each group (see Table 1): ND – non-diabetic group; D – diabetic group. Means that do not share a common letter are significantly different between groups ($p < 0.05$).

strated an important cytoprotective effect in both the liver and kidneys. This ability of BGEE to restore normal organ functions is supported by the finding that the general biochemical markers used for estimating liver and kidney functions, such as AST, ALT and CK enzymatic activities and creatinine and BUN levels were significantly lowered after the diabetic rats were administered BGEE. The observation that the treatment with BGEE increased the activity of the pro-survival Akt kinase and suppressed the activation of the downstream apoptosis effector caspase-3 suggests that BGEE shifted the balance between life and death signaling pathways in favor of the pro-survival route.

Earlier findings have questioned the scavenging activities of the β-glucans. Tsiapali et al. (2001) who examined the free radical scavenging activities of a variety of carbohydrate polymers concluded that the weak free radical scavenging activity

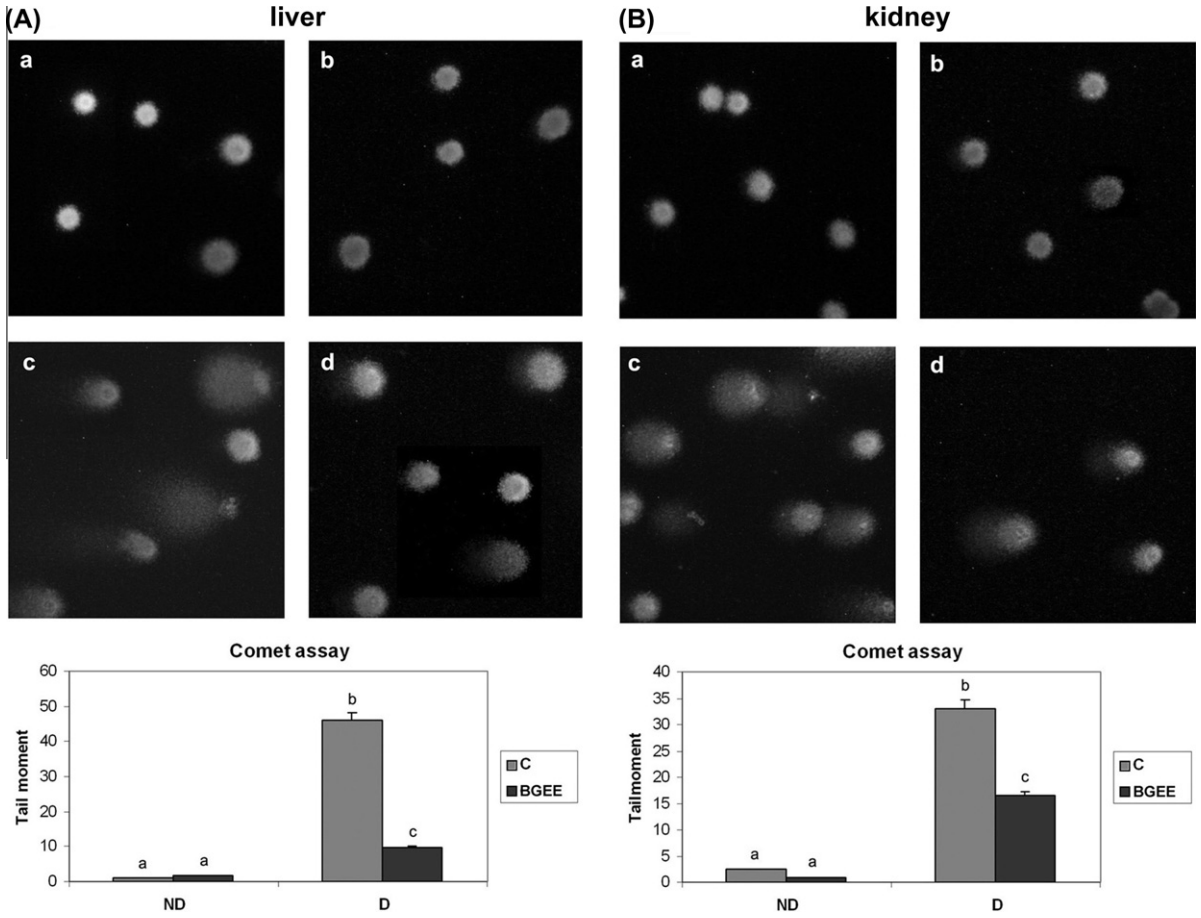

Fig. 4 – The effect of the treatment with BGEE on DNA damage in the liver (A) and kidneys (B) of non-diabetic and diabetic rats, estimated by the alkaline Comet assay. Representative images of comets obtained from: a – ND; b – BGEE-treated ND; c – D; d – BGEE-treated D rats. Assessment of DNA damage using the tail moments shown in the lower graph. Results are expressed as the means ± S.E.M from three separate experiments performed in triplicate. ND – non-diabetic group; D – diabetic group. Means that do not share a common letter are significantly different between groups ($p < 0.05$).

of the examined glucan polymers could not explain the observed modulatory effect on the inflammatory responses in tissue, culture and/or disease models of inflammation. Despite this result, increasing attention has been devoted to potential antioxidants of polysaccharide origin. Results that have been presented elsewhere (Kogan et al., 2005) and herein support the notion that the protective properties of the β-glucans could in part be ascribed to their free radical scavenging properties. β-Glucans with free radical scavenging properties have been identified in some cereals and legumes. As the major components of mushrooms, the β-glucans play an important role in the observed health-promoting properties through their ability to reduce blood cholesterol and blood glucose levels, as well as their free radical scavenging-activities (Lo & Wasser, 2011). The *in vitro* assays performed herein (DPPH radical scavenging activity, ferrous ions-chelating, hydrogen peroxide and nitric oxide radical scavenging capacity) lend further support for an antioxidant activity of BGEE. This result reflects the findings that an antioxidant property of β-glucans protects macrophages from ionizing radiation, restores bone marrow production and generally protects animals from the lethal effects of irradiation (Kogan et al., 2005). The most often applied and investigated natural antioxidants are ascorbic acid, α-tocopherol and mannitol. Evidence has been presented arguing that β-glucans are antioxidants whose scavenging ability is somewhere between that of α-tocopherol and the water-soluble antioxidant mannitol (Babincová, Bačová, Machová, & Kogan, 2002).

Glucose oxidation is believed to be the main source of free radicals in diabetes and hyperglycemia-induced oxidative stress has been implicated in the pathophysiology of diabetic complications. The observed ability of BGEE to lower hyperglycemia is an important aspect of its positive biological effect (Table 1). Our results mirror the findings obtained previously in animal experiments and clinical trials describing the reduction of blood glucose levels by fungal and oat β-glucan preparations (Lo, Tsai, Wasser, Yang J.G., & Huang, 2006). Our results also show that the treatment with BGEE lowers the serum iron and ferrous ion levels which are increased in diabetic rats. The toxicity of iron arises from its ability to catalyze the formation of ROS (Fernandez-Real, Lopez-Bermejo, & Ricart, 2005). The antioxidant effects of the BGEE we observed *in vitro* were also confirmed in *in vivo* experiments performed on liver and kidneys of diabetic rats. ROS

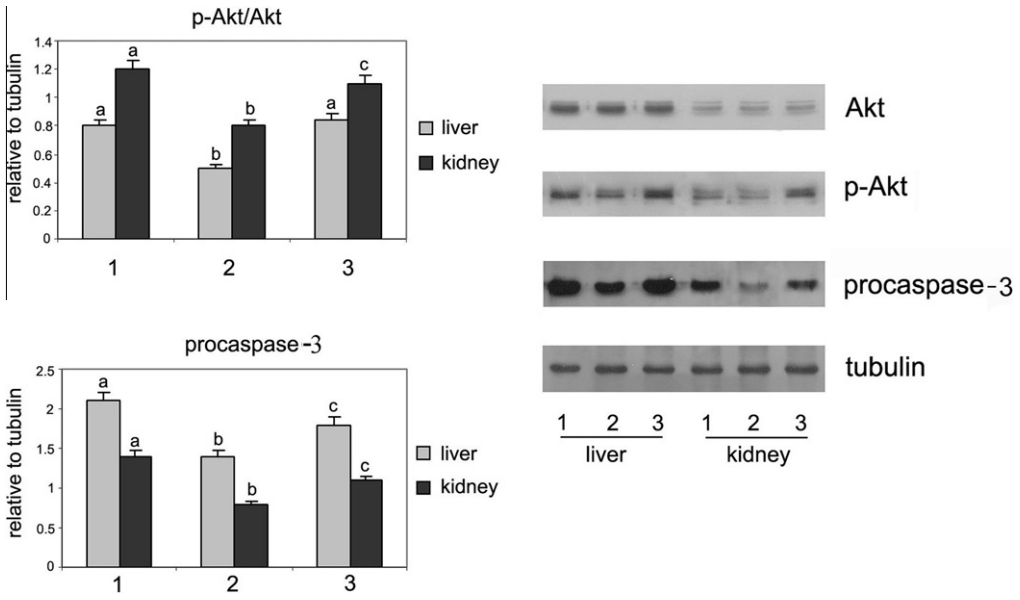

Fig. 5 – The effects of BGEE administration on the relative protein levels of Akt kinase, phosphorylated, active Akt kinase (p-Akt), and pro-caspase-3 in the liver and kidneys of non-diabetic and diabetic rats. Immunoblot analysis of the rat liver and kidney homogenate was performed with anti-Akt, anti-p-Akt and anti-caspase-3 antibodies. Tubulin was used as an internal control. Lanes 1, 2 and 3 – liver homogenates; 4, 5 and 6 – kidney homogenates. Lanes 1 and 4 – non-diabetic rats; lanes 2 and 5 – diabetic (D) rats, lanes 3 and 6 – BGEE-treated D animals. Immunoblot analysis was quantified using TotalLab (Phoretix) electrophoresis software (ver. 1.10; relative to tubulin) and is presented on the graphs. The values are means ± S.E.M. from three separate experiments. $^{*}p < 0.05$ statistically significant difference versus non-diabetic control. Means that do not share a common letter are significantly different between groups ($p < 0.05$).

play a crucial role in the induction and progression of liver disease via cumulative molecular damage that leads to cell dysfunction (Lin et al., 2009). In the kidneys, increased glucose uptake promotes the formation of AGE which in turn act as "secondary" inducers of ROS and generators of oxidative stress (Bhatti et al., 2005). Our results show that BGEE administration lowered the levels of lipid peroxidation in both the liver and kidneys (Fig. 2A and B). After the treatment of diabetic rats with BGEE we observed significantly improved activities of the antioxidative enzymes SOD and to a lesser extent of CAT in the liver. In the kidneys, the activities of total SOD and CuZnSOD were fully restored to the normal levels and of MnSOD to 50% above the control level of activity. In the kidneys as in the liver, CAT activity remained more impervious to improvement (Fig. 3). Consequently, we assumed that as a result of enhanced antioxidant enzyme activities, the levels of DNA damage in the liver and kidneys would be decreased. Indeed, this was confirmed by the comet assay (Fig. 4). These results are consonant with the recent finding that β-glucans do not exert either genotoxic or mutagenic effects while protecting against DNA damage by ROS capture (Angeli, Ribeiro, Bellini, & Mantovani, 2009). A number of "functional foods", including those that contain β-glucans, have recently been shown to protect human DNA against genotoxic effects, associated development of cancer and other chronic diseases (Slameňová, Kováčiková, Horváthovaá, Wsólová, & Navarová, 2010).

Tissue homeostasis is dependent on the balance between signals that control cell proliferation and signals determining cell death. Martin et al. (2002) showed that strong oxidants that generate intracellular ROS lead to Akt kinase downregulation (by dephosphorylation), and to caspase-3 activation (by proteolysis of its inactive form pro-caspase-3). We showed that the treatment of diabetic rats with BGEE which displayed *in vitro* and *in vivo* antioxidant capacities, promoted a decrease in DNA damage in the liver and kidneys. The observed decrease in DNA damage correlated with increased Akt kinase activity and decreased pro-caspase-3 degradation. One of the most actively studied kinase pathways in basic research and drug development includes the serine–threonine kinase Akt. Akt is a downstream target of phosphatidylinositol-3 (PI-3) kinase, and it plays a central role in signals that mediate cell growth and cell survival. It is widely accepted that the activation of Akt kinase exerts a pro-survival effect in cells. Stimulation of Akt by phosphorylation inhibits cell death in part by phosphorylation of Bcl-2-associated death promoter (BAD) protein which then releases Bcl-2 to inhibit apoptosis, as well as by inhibiting the caspase activation cascade, i.e. activation of the apoptotic effector caspase-3 (Hsu, Lee, & Lin, 2002). Caspase-3 is responsible for the cleavage of DNA repair enzymes, DNA fragmentation factor, nuclear structural proteins and cytoskeletal proteins. Several different types of antioxidants are capable of decreasing caspase-3-mediated apoptosis and reducing tissue injury (Lin et al., 2009). Hsu et al. (2002) have shown that the β-glucan from *Ganoderma lucidum* exerts an anti-apoptotic effect on neutrophils through the Akt-regulated signaling pathway and inhibition of pro-caspase-3 proteolysis. To our knowledge, we have presented herein for the first time *in vivo* evidence that was obtained on an animal model of diabetes, for β-glucan-in-

duced stimulation of Akt kinase activity and decreased caspase-3 activation.

5. Conclusion

Intake of β-glucans has been shown to reduce diabetes risk factors and to delay the onset of diabetic complications. Administration of BGEE to intact rats did not affect the examined biochemical parameters, confirming the safety of this commercially available BGEE product. *In vitro* and *in vivo* experiments suggest that significant free radical scavenging and antioxidant activities of BGEE were responsible for a systemic readjustment of major biochemical indicators and for a reduction of DNA damage in the liver and kidney of diabetic rats. Considering that BGEE-treated rats displayed increased Akt kinase activity and suppressed activation of the downstream apoptosis effector caspase-3 implies that activation of this prosurvival pathway plays a vital role in mediating the beneficial effects of the β-glucan. This study proves that BGEE has a significant impact on cytoprotection and that it promotes a systemic improvement in diabetic animals. These findings may be relevant with regard to the therapeutic potential of BGEE in treating the diabetic condition but also in the management of oxidative stress in other pathological states.

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