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Bee venom induced cytogenetic damage and decreased cell viability in human white blood cells after treatment *in vitro*: A multi-biomarker approach

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ABSTRACT

The aim of this study was to evaluate cytogenotoxic effects of bee venom to human lymphocytes and take a look into the mechanisms behind them. Bee venom was tested in concentrations ranging from 0.1 μg/ml to 20 μg/ml over different lengths of time. Cell viability, type of the cell death, and morphological alterations were evaluated using phase-contrast and fluorescent microscopy in addition to DNA diffusion assay, whereas cytogenotoxic effects were assessed with the micronucleus test. DNA damage and its relation to oxidative stress were evaluated combining the standard alkaline and the Fpg-modified comet assay. Our results showed lower cell viability, morphological cell alterations, cytogenotoxicity, and dominantly necrotic type of cell death in human lymphocytes after treatment with bee venom. All the effects were time- and dose-dependent. These results provide an insight into the effects of bee venom on the cell structure that could be relevant for therapeutic purposes.

1. Introduction

Bee venom from *Apis mellifera* is a complex mixture of at least 18 active components with a wide variety of properties. Bee venom has been used in oriental medicine to treat chronic inflammatory diseases such as rheumatoid arthritis and to relieve pain (Kwon et al., 2002; Kang et al., 2002; Son et al., 2007). In the past few years a number of studies have been published indicating its radioprotective, antimutagenic, anti-inflammatory, antinociceptive, and anticancer properties (Varanda and Tavares, 1998; Kim et al., 2003; Lee et al., 2004; Son et al., 2007; Gajski and Garaj-Vrhovac, 2009). In addition, recent studies have reported several mechanisms of cytotoxicity in different types of cancer cells such as cell cycle alterations, effect on proliferation and growth inhibition, as well as induction of apoptosis or necrosis (Liu et al., 2002; Jang et al., 2003; Hu et al., 2006; Moon et al., 2006; Ip et al., 2008a,b; Maher and McClean, 2008). However, little has been said about cytogenotoxic effects of whole bee venom on normal human cells (Lee et al., 2007; Stuhlmeier, 2007; Gajski and Garaj-Vrhovac, 2009).

The components of whole bee venom include melittin, apamin, secapin, procapine, adolapin, histamine, catecholamines, and mast cell degranulating peptide. Dominant enzymes are phospholipase A_2, and in much lower amounts hyaluronidase, acid phosphomonoesterase, lysophospholypase, and α-glucosidase. Bee venom also contains several physiologically active amines, glucose, fructose, and phospholipids, all having effects on many cellular systems (Neuman and Habermann, 1954; Habermann, 1972; Gauldie et al., 1976; Stuhlmeier, 2007).

Melittin, the main component and the principal toxin of the bee venom that accounts for around 50% of its dry weight is a highly basic polypeptide with molecular weight of 2850 Da (Neuman and Habermann, 1954; Vogel, 1981). Its known amino

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acid sequence consists of 26 amino acid residues (Gevod and Birdi, 1984; Dempsey, 1990). Melittin is mostly hydrophobic, but does have a hydrophilic sequence near the C-terminus. This polypeptide readily integrates into and disrupts both natural and synthetic phospholipid bilayers (Lauterwein et al., 1980; Lavialle et al., 1980). Melittin also enhances the activity of phospholipase A_2 and has a variety of effects on living cells (Lad and Shier, 1979). It is cytotoxic to haematopoietic cells lymphocytes, thymocytes, and erythrocytes, as well as to intestinal cells (Tosteson et al., 1985; Watala and Kowalczyk, 1990; Shaposhnikova et al., 1997; Maher and McClean, 2006; Garaj-Vrhovac and Gajski, 2009). In addition, it interacts with a broad range of metabolic functions in mammalian cells. Its toxic effect is based on the disruption of the plasma membrane and changes to the enzyme system, and its lytic activity is probably caused by its ability to insert into phospholipid layers (Vogel, 1981; Fletcher and Jiang, 1993).

Seeing the wide range of effects of the whole bee venom, we wanted to take a careful look at the cytogenotoxic effects induced by this complex mixture and mechanisms behind these effects. To do that, we employed several methods which are sensitive for different types of cytogenetic alterations produced by physical and chemical agents (Fenech and Morley, 1985; Singh et al., 1988; Duke and Cohen, 1992; Singh, 2000; Umegaki and Fenech, 2000; Fenech et al., 2003; Lee et al., 2004; Singh, 2005; Collins et al., 2008, Kumaravel et al., 2009). We hoped that using them in combination might improve cell assessment, as these methods make it possible to evaluate cytotoxic effects even at the level of primary DNA damage or to establish the dynamics of its repair after exposure to cytotoxic or genotoxic agents (Liu et al., 2003; Gajski et al., 2008). We used the DNA diffusion assay, phase-contrast and fluorescent microscopy to see the impact on cell viability, type of cell death, and morphological alterations in peripheral blood lymphocytes, whereas the cytogenotoxic status was assessed with the micronucleus test. DNA damage and its relation to oxidative stress were evaluated using a combination of standard alkaline and Fpg-modified version of the comet assay after bee venom treatment. Bee venom was tested *in vitro* in concentrations ranging from 0.1 μg/ml to 20 μg/ml at different time periods from 1 h to 48 h.

2. Materials and methods

2.1. Blood sampling and experimental design

The effects of whole bee venom were evaluated in human peripheral blood lymphocytes obtained from a young (age 27 years), healthy, non-smoking, male donor. The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes or to known genotoxic chemicals that might have interfered with the results of the testing for a year before blood sampling. Blood was drawn by antecubital venipuncture into heparinised vacutainers containing lithium heparin as anticoagulant (Becton Dickinson, Franklin Lakes, NJ) under aseptic conditions. Lyophilized whole bee venom was purchased from Sigma (St Louis, MO). Just before the beginning of the experiment, bee venom was dissolved in sterile redistilled water at 25 °C. The aliquot of bee venom water solution was added to the whole blood samples to make the final concentrations of 0.1 μg/ml to 20 μg/ml for 1 h, 6 h, 24 h, or 48 h. Unexposed control samples for each exposure times were also included. Blood samples were incubated *in vitro* at 37 °C in a humified atmosphere with 5.0% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). After the treatment, all experiments were conducted according to the standard protocols listed below. All experiments were done in triplicate and results are presented as mean values ± SE (standard error of the mean).

2.2. Determination of morphological changes and cell viability

Peripheral blood lymphocytes were treated with whole bee venom over different lengths of time. Lymphocytes were isolated using a modified Ficoll-Histopaque (Sigma) centrifugation method (Singh, 2000). Cell viability was determined by differential staining with acridine orange and ethidium bromide and by fluorescence microscopy (Duke and Cohen, 1992). The slides were prepared using 200 μl of human peripheral blood lymphocytes and 2 μl of stain (acridine orange and ethidium bromide, both diluted in phosphate-buffered saline, PBS). A total of 100 cells per repetition were examined with an Olympus AX-70 microscope (Tokyo, Japan), using a 60× objective and fluorescence filters of 515–560 nm. The cells were divided into two categories: live cells with a functional membrane and with uniform green staining of the nucleus and dead cells with uniform red staining of the nucleus. To determine the morphological changes, phase-contrast microscopy was used. Cells were examined with a Nikon Eclipse E400 POL microscope (Tokyo, Japan), using a 40× objective.

2.3. DNA diffusion assay

DNA diffusion assay was used to determine the type of cell death, following the protocol described by Singh (2000). Chemicals were provided by the Sigma Chemical Company. Agarose-precoated slides were made by spreading 50 μl of 0.7% normal melting agarose on each slide and drying them at room temperature. Microgels were made on agarose-precoated slides by mixing 5 μl of whole blood culture with 50 μl of 0.7% high-resolution agarose and pipetting it onto the slide. The gel was immediately covered with a cover glass. The slides were coded and cooled on ice for 1 min. The cover glasses were removed, and 200 μl of 2% agarose solution was layered. After keeping the slides for 1 min on ice, the cover glasses were removed and the slides were immersed in a freshly made lysing solution (1.25 M NaCl, 1 mM tetra sodium EDTA, 5 mM Tris–HCl pH 10, 0.01% sodium lauroyl sarcosine, 0.2% DMSO freshly added, 300 mM NaOH freshly added) for 10 min at room temperature. After lysis, the slides were twice immersed in a neutralizing solution (50% ethanol, 1 mg/ml spermine, 20 mM Tris–HCl pH 7.4) for 30 min at room temperature, then air-dried and stored at room temperature. They were stained with ethidium bromide (20 μg/ml) and covered with a coverslip for 10 min. One thousand lymphocytes per slide were analyzed under the fluorescent microscope (Zeiss, Oberkochen, Germany) using a 60× objective equipped with 515–560 nm excitation filters and a 590 nm barrier filter.

Lymphocytes undergoing apoptosis or necrosis were distinguished from normal cells in accordance with figures and instructions given by Singh (2005). Apoptotic cell nuclei have a hazy or undefined outline without any clear boundary due to nucleosomal-sized DNA diffusing into the agarose. Necrotic cell nuclei are bigger and are poorly defined. They have a clearly defined outer boundary of the DNA halo and a relatively homogeneous halo appearance.

2.4. *Alkaline comet assay*

To measure DNA damage after treatment with whole bee venom the comet assay was carried out under alkaline conditions, basically as described by Singh et al. (1988). Fully frosted slides were covered with 1% normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off the slide. The slides were then coated with 0.6% NMP agarose. When this layer had solidified, a second layer containing 5 μl of whole blood sample mixed with 0.5% low melting point (LMP) agarose (Sigma) was placed on the slides. After 10 min of solidification on ice, the slides were covered with 0.5% LMP agarose. The slides were then immersed for 1 h in ice cold freshly prepared lysis solution [2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate (Sigma), pH 10] with 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Kemika, Zagreb, Croatia) (added immediately prior to use) to lyse the cells and allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH 13.0) and the slides were placed in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4 °C under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA). After electrophoresis the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. The slides were then stored in sealed boxes at 4 °C until analysis.

2.5. *Fpg-modified comet assay*

The analysis of oxidized purines was performed using a modified Fpg FLARE™ assay kit (Trevigen Inc, Gaithersburg, USA). Fully frosted microscopic slides were prepared. Each was cov-

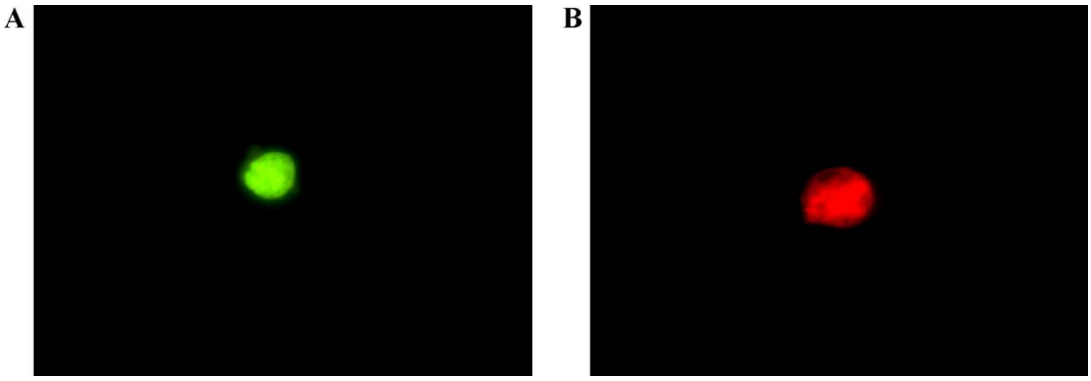

Fig. 1 – Cell viability microphotographs represent viable lymphocyte from the un-exposed sample (A), and dead (B) cell from sample treated with whole bee venom. Cells were differentially stained with acridine orange and ethidium bromide. Cells were photographed under the fluorescent microscope, using a 60× objective and fluorescence filters of 515–560 nm.

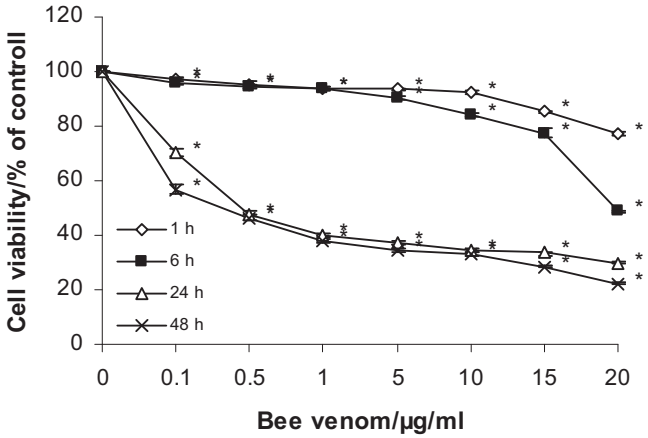

Fig. 2 – Cell viability after *in vitro* treatment of human peripheral blood lymphocytes with whole bee venom in different concentrations over different lengths of time. Viability was determined by acridine orange and ethidium bromide staining, using fluorescence microscopy at the indicated time points. Results are presented as mean values ± SE (standard error of the mean). *Statistically significant decrease compared to the corresponding control ($P < 0.05$).

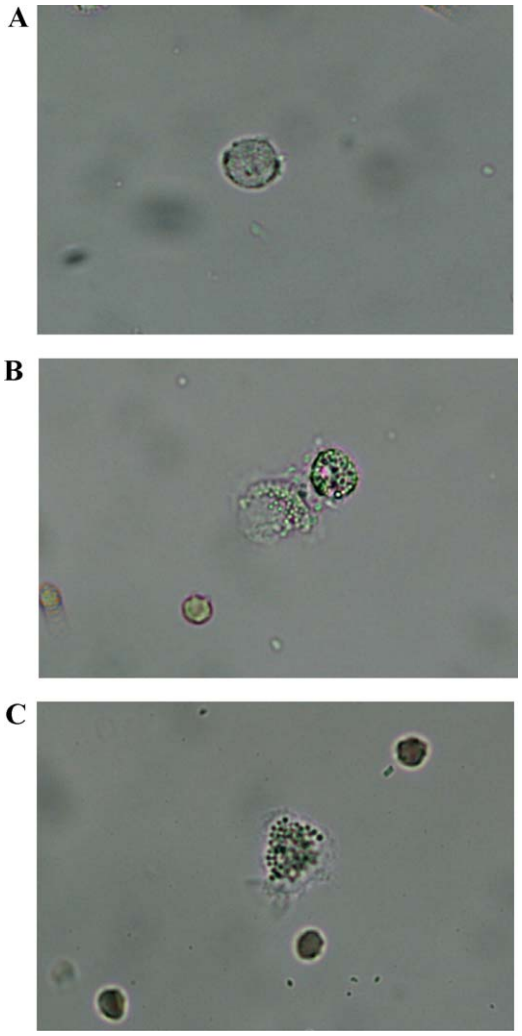

Fig. 3 – Changes in the morphology of peripheral blood human lymphocytes after treatment with whole bee venom. Human lymphocytes were incubated at 37 °C for 1 h and 24 h with 1 μg/ml and 10 μg/ml of whole bee venom. Viable intact cells in the control sample (A), whole bee venom induced cellular membrane alterations and granulation of the cells (B) and cell lysis in human lymphocytes (C). Cells were photographed under the phase-contrast microscope using a 40× objective.

ered with 1% normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off the slides and they were then coated with 0.6% NMP agarose. LMP agarose was melted and stabilized in a water bath at 37 °C. For each sample and control, 5 μl of whole blood was mixed with 100 μl of LMP agarose (provided with the FLARE™ assay kit) and placed on the slides. After 10 min of solidification on ice, the slides were covered with 0.5% LMP agarose and then immersed in a pre-chilled lysis solution (provided with the FLARE™ assay kit) and kept in a refrigerator at 2 °C for 60 min, followed by immersion in the FLARE™ buffer, three times for 15 min. After lysis, the slides were treated with 100 μl of Fpg enzyme [1:500 in REC dilution buffer (provided with the FLARE™ assay kit)]. The enzyme was diluted right before use. Control slides were treated with 100 μl of REC dilution buffer only. The slides were placed horizontally in a humid chamber at 37 °C for 30 min. All slides were then immersed in an alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) for 40 min followed by electrophoresis in a pre-chilled alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) at 1 V/cm for 20 min. After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. Slides were stored at 4 °C in sealed boxes until analysis.

2.6. *Image analysis*

One hundred randomly captured comets from each slide were examined at 250× magnification using an epifluorescence

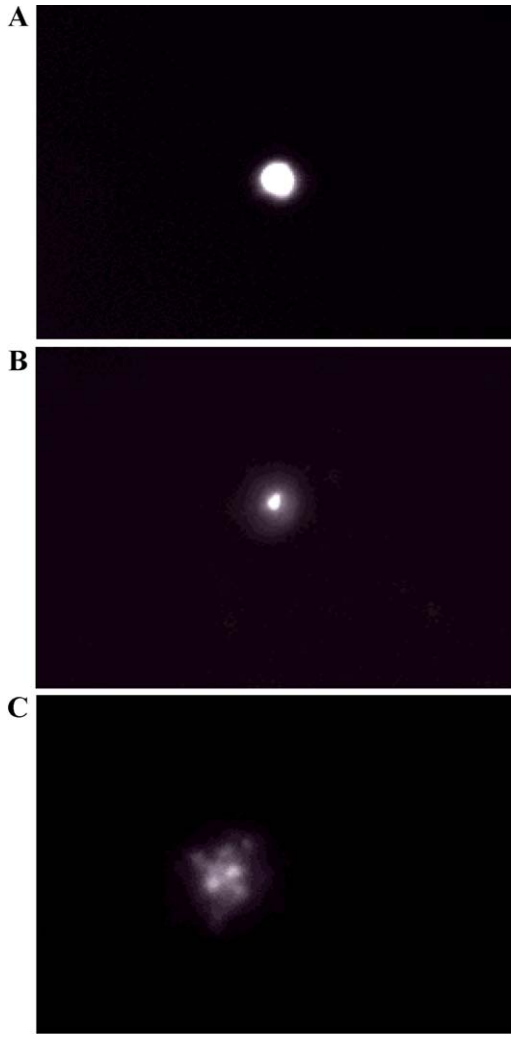

Fig. 4 – DNA diffusion microphotographs represent viable lymphocyte from the un-exposed sample (A), and apoptotic (B) and necrotic (C) cells from samples treated with whole bee venom. Cells were stained with ethidium bromide. Cells were photographed under the fluorescent microscope using a 60× objective equipped with a 515–560 nm excitation filters and a 590 nm barrier filter.

microscope (Zeiss, Oberkochen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, Haverhill, Suffolk, UK). An automated image analysis system was used to acquire images, compute the integrated intensity profile for each cell, estimate the comet cell components and evaluate the range of derived parameters. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets or comets without distinct head ("clouds", "hedgehogs", or "ghost cells"). To quantify DNA damage, the following comet parameters were evaluated: tail length, tail intensity (percentage of DNA), and tail moment.

2.7. Cytokinesis-blocked micronucleus (CBMN) test

To evaluate further cell disturbances after whole bee venom treatment the micronucleus test was performed in agreement with guidelines described by Fenech and Morley (1985). After the exposure to bee venom samples were centrifuged, washed in medium, centrifuged, supernatant removed and pellet was incubated in a Euroclone medium (Chromosome kit P, Euroclone, Milano, Italy) at 37 °C in an atmosphere of 5% CO_2 for 72 h. Cytochalasin-B (Sigma) at the final concentration of 6 μg/ml was added to each sample on the 44th hour of incubation, and the cells were harvested after another 28 h. After adding saline, cells were fixed with a cold fixative methanol:acetic acid (3:1, v/v). The cells were resuspended in a small volume of fixative solution and dropped onto slides and stained Giemsa dye (Merck, Darmstadt, Germany). We followed the criteria for the selection of binucleated cells and identification according to the HUMN project. Each sample was analyzed for the total number of micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NB) per 500 binucleated cells according to the criteria previously published by Fenech et al. (2003). The frequency of binucleated cells containing one or more MN was also determined. Only binucleated cells with well-preserved cytoplasm were scored for MN analysis. Microscopic analysis was done under a light microscope with a 40 × 10 magnification.

2.8. Statistical analysis

The comet parameters of tail length, tail intensity, and tail moment measured after treatment with whole bee venom was evaluated using Statistica 5.0 package (StaSoft, Tulsa, OK). Each sample was characterized for the extent of DNA damage by considering the mean ± SE (standard error of the mean). In order to normalize the distribution and to equalize the variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post hoc analyses of differences were done by using the Scheffé test. As for the cell viability, statistical significance was analyzed using the Student's *t*-test. The significance of MN parameters was tested using the χ^2-test. The level of statistical significance was set at $P < 0.05$.

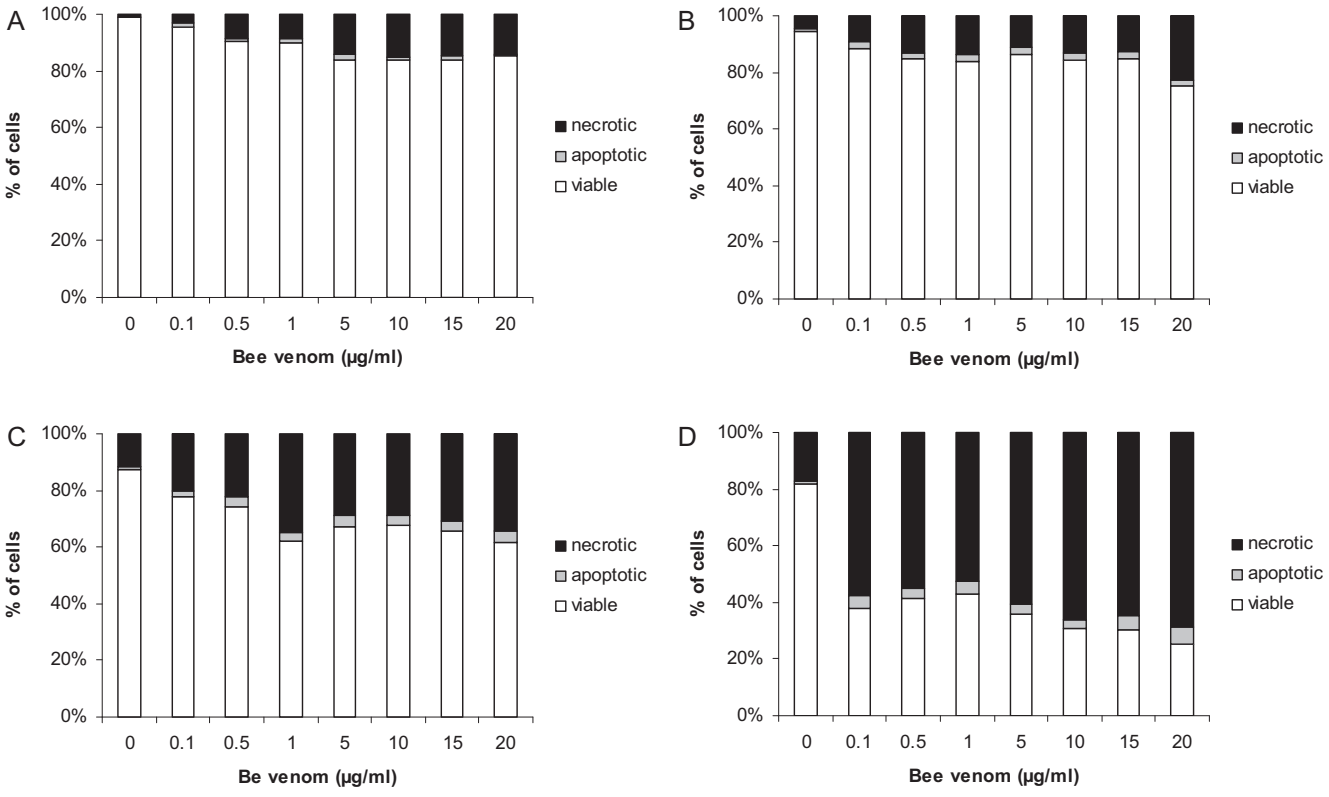

Fig. 5 – Percentage of apoptotic and necrotic cells in human peripheral blood lymphocytes after treatment with whole bee venom at different concentrations and time periods of 1 h (A), 6 h (B), 24 h (C), and 48 h (D).

3. Results

3.1. Determination of morphological changes and cell viability

In order to investigate the effects of bee venom on the viability of peripheral blood lymphocytes cells were treated with whole bee venom in different concentrations over different lengths of time. Changes were determined according to the different staining of the nucleus (Fig. 1). As shown in Fig. 2, viability of the cells decreased in time- and dose-dependent manner. Bee venom also induced morphological changes of cell membrane, granulation, and lysis (Fig. 3).

3.2. DNA diffusion assay

Changes were determined according to cell appearance (Fig. 4). Fig. 5 shows the percentage of apoptotic and necrotic cells determined by the diffusion test. Bee venom increased both apoptosis and necrosis in respect to control cells, and the percentage was time- and dose-dependent. Necrosis, however, dominated.

3.3. Alkaline and Fpg-modified comet assay

Tables 1 and 2 show the basic statistics for the tail length, tail intensity, and tail moment obtained with the alkaline and Fpg-modified comet assay at different concentrations and times of exposure. All three parameters significantly deviated from the normal distribution in both assays. The differences between control and treated cells were significant and were time- and dose-dependent ($P < 0.05$). They were even greater when the Fpg-modified comet assay was applied. These findings suggest that the Fpg-modified comet assay is more sensitive to whole bee venom effects than the standard comet assay.

3.4. Cytokinesis-blocked micronucleus (CBMN) test

Using the cytokinesis-blocked micronucleus test (CBMN), we evaluated MN, NPB, and NB frequency. The total number of MN and their distribution are summarized in Table 3. All three parameters of the CBMN test increased with the time of expo-

Table 1 – Alkaline comet assay parameters (tail length, tail intensity and tail moment) in human peripheral blood lymphocytes after treatment with whole bee venom at different concentrations and time periods.

Exposure time (h)	Concentration (μg/ml)	Tail length (μm) Mean ± SE	Tail intensity Mean ± SE	Tail moment Mean ± SE
1	Control	13.67 ± 0.13	1.28 ± 0.15	0.16 ± 0.02
	0.1	15.42 ± 0.25*	2.53 ± 0.28	0.32 ± 0.04
	0.5	16.21 ± 0.26*	2.58 ± 0.32	0.35 ± 0.04
	1	17.40 ± 0.30*	2.56 ± 0.25	0.38 ± 0.04
	5	18.29 ± 0.39*	3.18 ± 0.31*	0.46 ± 0.04*
	10	18.69 ± 0.43*	3.40 ± 0.40*	0.50 ± 0.06*
	15	18.28 ± 0.39*	3.44 ± 0.40*	0.50 ± 0.06*
	20	19.08 ± 0.40*	3.39 ± 0.40*	0.52 ± 0.06*
6	Control	14.32 ± 0.17	1.43 ± 0.18	0.18 ± 0.22
	0.1	15.88 ± 0.31*	2.52 ± 0.35*	0.34 ± 0.05*
	0.5	16.68 ± 0.31*	2.62 ± 0.30*	0.36 ± 0.04*
	1	17.49 ± 0.35*	2.72 ± 0.36*	0.38 ± 0.05*
	5	18.71 ± 0.46*	3.41 ± 0.46*	0.51 ± 0.07*
	10	18.91 ± 0.47*	3.79 ± 0.44*	0.56 ± 0.06*
	15	18.76 ± 0.44*	3.27 ± 0.41*	0.50 ± 0.06*
	20	18.47 ± 0.47*	4.32 ± 0.50*	0.63 ± 0.08*
24	Control	14.65 ± 0.22	2.19 ± 0.21	0.27 ± 0.03
	0.1	21.47 ± 0.65*	6.81 ± 0.68*	1.04 ± 0.11*
	0.5	25.41 ± 1.00*	7.70 ± 0.68*	1.27 ± 0.12*
	1	27.37 ± 0.87*	7.82 ± 0.67*	1.37 ± 0.12*
	5	29.69 ± 1.20*	8.87 ± 0.75*	1.63 ± 0.14*
	10	30.29 ± 1.21*	7.03 ± 0.67*	1.33 ± 0.13*
	15	28.24 ± 1.17*	7.83 ± 0.77*	1.37 ± 0.14*
	20	30.66 ± 1.55*	7.35 ± 0.72*	1.52 ± 0.17*
48	Control	16.69 ± 0.24	2.14 ± 0.20	0.27 ± 0.03
	0.1	25.91 ± 1.14*	8.02 ± 0.73*	1.44 ± 0.15*
	0.5	27.97 ± 1.30*	8.34 ± 0.74*	1.50 ± 0.15*
	1	28.66 ± 1.54*	7.83 ± 0.93*	1.52 ± 0.19*
	5	29.76 ± 1.54*	8.52 ± 0.87*	1.66 ± 0.19*
	10	34.80 ± 1.78*	11.60 ± 1.09*	2.41 ± 0.24*
	15	33.17 ± 1.33*	12.56 ± 1.06*	2.42 ± 0.21*
	20	36.56 ± 1.73*	13.62 ± 1.22*	2.76 ± 0.25*

* Statistically significant compared to corresponding control ($P < 0.05$).

Table 2 – Fpg-modified comet assay parameters (tail length, tail intensity and tail moment) in human peripheral blood lymphocytes after treatment with bee venom at different concentrations and time periods.

Exposure time (h)	Concentration (μg/ml)	Tail length (μm) Mean ± SE	Tail intensity Mean ± SE	Tail moment Mean ± SE
1	Control	15.65 ± 0.18	2.59 ± 0.32	0.34 ± 0.04
	0.1	17.40 ± 0.23**	4.35 ± 0.46**	0.61 ± 0.06**
	0.5	18.30 ± 0.32*,**	4.71 ± 0.52**	0.68 ± 0.07**
	1	19.65 ± 0.51*,**	5.19 ± 0.59**	0.79 ± 0.09*,**
	5	21.44 ± 0.46*,**	5.26 ± 0.53*,**	0.85 ± 0.08*,**
	10	20.01 ± 0.47*,**	5.98 ± 0.60*,**	0.91 ± 0.09*,**
	15	20.84 ± 0.51*,**	5.91 ± 0.60*,**	0.89 ± 0.09*,**
	20	20.20 ± 0.61*,**	6.47 ± 0.75*,**	1.02 ± 0.12*,**
6	Control	15.79 ± 0.21	3.01 ± 0.40	0.41 ± 0.05
	0.1	24.65 ± 1.00*,**	8.12 ± 0.79*,**	1.53 ± 0.17*,**
	0.5	25.44 ± 0.97*,**	9.11 ± 0.66*,**	1.59 ± 0.13*,**
	1	29.41 ± 1.17*,**	10.95 ± 0.95*,**	2.26 ± 0.23*,**
	5	31.88 ± 1.23*,**	12.37 ± 0.91*,**	2.56 ± 0.21*,**
	10	31.14 ± 1.21*,**	13.55 ± 0.99*,**	2.85 ± 0.25*,**
	15	30.46 ± 1.06*,**	14.65 ± 0.93*,**	2.90 ± 0.21*,**
	20	30.92 ± 1.13*,**	13.37 ± 0.97*,**	2.73 ± 0.23*,**
24	Control	15.97 ± 0.36	3.34 ± 0.45	0.46 ± 0.06
	0.1	30.30 ± 1.29*,**	13.63 ± 1.15*,**	2.96 ± 0.29*,**
	0.5	32.99 ± 1.51*,**	14.75 ± 1.27*,**	3.55 ± 0.34*,**
	1	33.68 ± 1.49*,**	15.16 ± 1.25*,**	3.64 ± 0.35*,**
	5	37.75 ± 1.49*,**	17.31 ± 1.28*,**	4.64 ± 0.39*,**
	10	39.63 ± 1.70*,**	20.03 ± 1.32*,**	5.24 ± 0.42*,**
	15	39.95 ± 1.33*,**	19.05 ± 1.14*,**	4.94 ± 0.36*,**
	20	38.88 ± 1.44*,**	20.27 ± 1.33*,**	5.17 ± 0.41*,**
48	Control	16.54 ± 0.39	3.62 ± 0.50	0.52 ± 0.07
	0.1	32.99 ± 1.54*,**	13.45 ± 1.11*,**	3.42 ± 0.34*,**
	0.5	34.57 ± 1.63*,**	14.89 ± 1.35*,**	3.97 ± 0.42*,**
	1	36.50 ± 1.79*,**	17.79 ± 1.61*,**	5.06 ± 0.54*,**
	5	37.51 ± 1.83*,**	18.67 ± 1.58*,**	5.16 ± 0.51*,**
	10	41.03 ± 1.85*,**	20.35 ± 1.54*,**	5.87 ± 0.50*,**
	15	42.96 ± 1.69*,**	22.15 ± 1.47*,**	6.29 ± 0.47*,**
	20	41.56 ± 1.69*,**	21.54 ± 1.43*,**	5.90 ± 0.45*,**

* Statistically significant compared to corresponding control ($P < 0.05$).
** Statistically significant compared to buffer control ($P < 0.05$).

sure to whole bee venom and were dose-dependent. All of them were significantly higher in the samples exposed to bee venom than in control samples ($P < 0.05$). Of the three parameters, total MN and NB frequency seem to be the best predictors of exposure to whole bee venom.

4. Discussion

Utilization of venoms of various organisms has a long history in traditional and alternative medicine (Harvey, 1998; Lewis and Garcia, 2003). In recent years science has shown a growing interest in the application of venom derived from *Apis mellifera* (Son et al., 2007). Our results have demonstrated that bee venom changes cell membrane and shape and causes their granulation. In addition, it kills cells in a dose- and time-dependent manner. Similar observations were made by Lee et al. (2007) in their comparative study on normal human lymphocytes versus human lymphoma HL-60 cells. They also noticed that bee venom decreased viability of human lymphocytes and inhibited proliferation. The same was observed in our previous study (Gajski et al., 2011), where we used several types of tumour cells (human laryngeal HEp-2 and cervical carcinoma HeLa cells and their drug resistant sublines, breast adenocarcinoma MCF-7 cells, colon adenocarcinoma SW620 cells, and glioblastoma A1235 cells) and non-tumour cell lines (human embryonic kidney HEK-293 cells and normal Hef fibroblasts). Decreased viability was more prominent in tumour than in non-tumour cells.

Lee et al. (2007) also noticed cell membrane blebbing. Stuhlmeier (2007) reported severe disruption of the cell membrane. Exposure of human fibroblast-like synoviocytes and red blood cells to concentrations of bee venom higher than 10 μg/ml resulted in nearly instantaneous loss of membrane integrity. More importantly, bee venom also induced profound membrane leakage, and where melittin was used instead of bee venom, approximately one-half of the concentration was needed to obtain very similar effects. The effects were clearly dose- and time-dependent. During lymphocyte microisolation we also noticed that the colour of the separation medium changed due to haemolysis. These effects can largely be attributed to the bee venom main component melittin. This peptide has a highly cytotoxic effect on different types of cells tested and it mainly disrupts cell membrane. It shares

Table 3 – Parameters of micronucleus test (micronuclei, nucleoplasmic bridges and nuclear buds) in human peripheral blood lymphocytes after whole bee venom treatment. Incidence of micronuclei, nucleoplasmic bridges and nuclear buds was evaluated by analyzing 500 binucleated cells.

Exposure time (h)	Concentration (μg/ml)	Micronuclei				Nucleoplasmic bridges		Nuclear buds		
		1MN	2MN	3MN	4MN	1NPB	2NPB	1NB	2NB	3NB
1	Control	1.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.1	4.00 ± 1.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 0.67*	0.33 ± 0.33	0.00 ± 0.00
	0.5	4.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	1.33 ± 0.33	0.00 ± 0.00	5.67 ± 1.20*	0.00 ± 0.00	0.00 ± 0.00
	1	3.67 ± 0.67	0.67 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	1.67 ± 0.67	0.00 ± 0.00	5.33 ± 0.88*	0.67 ± 0.67	0.00 ± 0.00
	5	6.67 ± 1.20*	1.67 ± 0.33	1.33 ± 0.33	0.00 ± 0.00	1.67 ± 1.20	0.00 ± 0.00	6.33 ± 0.33*	0.67 ± 0.33	0.00 ± 0.00
	10	6.67 ± 0.67*	1.33 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	2.33 ± 0.88*	0.00 ± 0.00	6.33 ± 0.33*	0.33 ± 0.33	0.33 ± 0.33
	15	11.33 ± 0.33*	3.33 ± 0.88*	1.67 ± 0.33	0.67 ± 0.67	1.67 ± 0.33	0.00 ± 0.00	4.67 ± 0.67*	1.33 ± 0.33	0.00 ± 0.00
	20	15.33 ± 1.45*	4.67 ± 0.33*	3.33 ± 0.33*	2.33 ± 0.33*	0.33 ± 0.33	0.00 ± 0.00	3.67 ± 1.76*	0.33 ± 0.33	0.00 ± 0.00
6	Control	1.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00
	0.1	8.33 ± 1.45*	1.33 ± 0.88	0.00 ± 0.00	0.33 ± 0.33	3.33 ± 0.33*	0.00 ± 0.00	5.67 ± 0.67*	0.33 ± 0.33	0.00 ± 0.00
	0.5	8.67 ± 0.33*	2.33 ± 0.33*	3.33 ± 0.33*	0.33 ± 0.33	2.67 ± 0.33*	0.00 ± 0.00	7.67 ± 0.67*	0.67 ± 0.67	0.00 ± 0.00
	1	7.33 ± 3.18*	1.33 ± 0.33	0.33 ± 0.33	3.67 ± 0.33*	4.33 ± 0.88*	0.00 ± 0.00	9.67 ± 1.76*	1.33 ± 0.33	0.33 ± 0.33
	5	10.33 ± 0.88*	3.67 ± 1.20*	2.33 ± 0.67*	0.67 ± 0.67	4.67 ± 1.20*	0.33 ± 0.33	8.33 ± 0.33*	0.33 ± 0.33	0.33 ± 0.33
	10	12.33 ± 0.88*	3.67 ± 1.20*	2.33 ± 0.67*	2.33 ± 0.33*	2.33 ± .033*	0.33 ± 0.33	9.33 ± 0.33*	1.33 ± 0.33	0.33 ± 0.33
	15	9.67 ± 1.20*	3.33 ± 0.33*	1.33 ± 0.33	2.67 ± 0.67*	1.33 ± 0.33	0.00 ± 0.00	13.33 ± 0.88*	1.67 ± 0.67	0.67 ± 0.67
	20	18.33 ± 0.88*	5.67 ± 1.20*	4.33 ± 0.33*	1.67 ± 0.67	2.33 ± 0.88*	0.00 ± 0.00	7.67 ± 2.33*	2.33 ± 0.33*	0.00 ± 0.00
24	Control	3.33 ± 0.88	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 0.67	0.33 ± 0.00	0.00 ± 0.00
	0.1	12.33 ± 0.33*	3.67 ± 0.33*	3.33 ± 0.33*	1.33 ± 0.33	3.67 ± 0.67*	0.00 ± 0.00	15.33 ± 0.33*	3.33 ± 0.33*	0.33 ± 0.33
	0.5	13.67 ± 0.67*	4.33 ± 1.45*	1.33 ± 0.33	0.67 ± 0.67	2.67 ± 0.67*	0.00 ± 0.00	14.33 ± 0.33*	1.33 ± 0.33	0.00 ± 0.00
	1	13.33 ± 0.88*	2.67 ± 1.20*	3.33 ± 0.33*	0.00 ± 0.00	2.67 ± 0.33*	0.00 ± 0.00	13.33 ± 1.45*	6.33 ± 0.88*	0.33 ± 0.33
	5	12.33 ± 0.88*	6.67 ± 0.33*	1.67 ± 0.33	0.67 ± 0.33	3.67 ± 0.67*	0.00 ± 0.00	11.33 ± 0.33*	1.67 ± 0.67*	0.33 ± 0.33
	10	14.33 ± 0.88*	6.33 ± 2.03*	2.33 ± 0.33*	1.67 ± 0.33	2.33 ± 0.33*	0.00 ± 0.00	13.33 ± 0.33*	5.33 ± 0.88*	1.67 ± 0.67
	15	11.67 ± 0.67*	3.67 ± 0.67*	0.67 ± 0.67	0.00 ± 0.00	3.67 ± 0.67*	0.67 ± 0.67	13.33 ± 0.33*	7.67 ± 0.33*	1.67 ± 0.67
	20	12.67 ± 0.67*	3.33 ± 0.33*	0.67 ± 0.67	0.67 ± 0.67	4.33 ± 0.33*	0.00 ± 0.00	14.33 ± 0.33*	6.33 ± 0.88*	1.33 ± 0.33
48	Control	1.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00
	0.1	12.33 ± 1.45*	2.67 ± 0.67*	1.33 ± 0.33	0.33 ± 0.33	3.33 ± 0.33*	0.00 ± 0.00	10.67 ± 0.67*	3.67 ± 0.67*	0.67 ± 0.33
	0.5	13.67 ± 1.20*	3.33 ± 0.88*	2.33 ± 0.67*	0.33 ± 0.33	1.33 ± 0.88	0.00 ± 0.00	9.67 ± 1.20*	4.33 ± 0.33*	0.00 ± 0.00
	1	15.67 ± 1.20*	6.33 ± 0.33*	1.33 ± 0.33	0.33 ± 0.33	1.67 ± 0.67	0.00 ± 0.00	13.67 ± 0.33*	5.33 ± 0.33*	0.67 ± 0.67
	5	16.67 ± 0.33*	3.67 ± 0.33*	0.00 ± 0.00	0.67 ± 0.67	3.33 ± 0.33*	0.33 ± 0.33	17.33 ± 0.88*	4.67 ± 0.67*	0.33 ± 0.33
	10	14.33 ± 1.45*	1.67 ± 1.20	0.33 ± 0.33	0.00 ± 0.00	3.33 ± 0.33*	0.00 ± 0.00	16.33 ± 1.45*	2.67 ± 0.67*	1.67 ± 0.33
	15	13.67 ± 0.67*	5.33 ± 0.33*	0.00 ± 0.00	0.00 ± 0.00	4.33 ± 0.33*	0.00 ± 0.00	11.67 ± 0.67*	3.33 ± 0.33*	1.33 ± 0.33
	20	12.67 ± 0.67*	2.67 ± 0.67*	1.33 ± 0.33	0.67 ± 0.33	3.67 ± 0.33*	0.00 ± 0.00	12.67 ± 1.20*	6.67 ± 0.33*	0.33 ± 0.33

MN, micronuclei; NPB, nucleoplasmic bridges; NB, nuclear buds.

* Statistically significant compared to corresponding control ($P < 0.05$).

amphipathic properties with a series of peptides that affect cell membrane bilayer integrity, either by defecting, disrupting, or forming pores in it (Stuhlmeier, 2007; Garaj-Vrhovac and Gajski, 2009).

Literature reports contradictory data about the type of cell death induced by bee venom. Bee venom caused apoptosis in mammary carcinoma cells, lung carcinoma cells, hepatoma cells, leukemic cells, cervical epidermoid carcinoma cells, synovial fibroblasts, vascular smooth muscle cells, and melanoma cells (Jang et al., 2003; Oršolić et al., 2003; Hong et al., 2005; Hu et al., 2006; Moon et al., 2006; Son et al., 2006; Ip et al., 2008b; Tu et al., 2008). In addition, recent reports refer to the necrosis of fibroblast-like synoviocytes, human cervical carcinoma HeLa cells, and Chinese hamster lung V79 fibroblasts (Stuhlmeier, 2007; Oršolić, 2009). Even though we too noticed an increase in apoptotic cells, necrosis was dominant. Similar results were reported by Stuhlmeier (2007) in which the majority of the DNA fragments form a pattern of necrotic DNA. Furthermore, Lee et al. (2007) observed neither sub-G1 fractions nor the cleavage of caspase-9, -3, or poly(ADP-ribose) polymerase (PARP) suggesting that bee venom induced cell death, but these cellular events were not accompanied by activation of the apoptotic machinery.

To see whether DNA damage could be affected by reactive oxygen species (ROS), we employed the Fpg-modified version of the comet assay, as enzymes such as formamidopyrimidine glycosilase can detect oxidative damage at the level of the DNA molecule caused by ROS (Collins et al., 2008; Gajski et al., 2008; Roberts et al., 2009). We found significant DNA migration and high percentage of tail DNA and increased tail moment. These results are in accordance with our previous study, where DNA damage, determined by the use of alkaline comet assay, was time- and dose-dependent (Gajski and Garaj-Vrhovac, 2008; Garaj-Vrhovac and Gajski, 2009). Lee et al. (2007) also found a similar effect. In their study, whole bee venom induced single-strand DNA breaks in human lymphocytes and HL-60 cells exposed to bee venom. This was also the case in another study by Ip et al. (2008a), where the comet assay showed DNA damage in human breast MCF7 cancer cells. All these parameters were even higher with the Fpg-modified assay, which points to an additional oxidative damage. Stuhlmeier (2007) found that bee venom induced reactive oxygen intermediates in mononuclear and polymorphonuclear cells. *In vivo*, large amounts of oxygen radicals released in response to bee venom might even amplify adverse effects of bee venom, for example, by increasing interleukin-8 production or activating collagenase (Weiss et al., 1985; DeForge et al., 1992; Stuhlmeier, 2007).

Our CBMN test results showed a significant increase in MN, NPB, and NB frequency and confirmed our earlier findings with higher concentrations of bee venom (Garaj-Vrhovac and Gajski, 2009). NPBs can be used as a measure of dicentric chromosomes (whose centromeres are being pulled apart to opposite poles of the cell) because single-strand and double-strand DNA breaks (and their misrepair and/or misreplication) induced by ROS are expected to result in the production of both acentric and dicentric chromosomes. In addition, acentric chromosomes (which lead to MN formation) are expected to be a by-product of dicentric chromosome formation (IAEA, 1986). Higher NPB in our study can therefore be related to oxidative stress (Umegaki and Fenech, 2000). Additionally, the MN test can detect NBs, which can measure the extent of chromosome rearrangement (Fenech et al., 2003; Kirsch-Volders et al., 2003; Thomas et al., 2003; Fenech, 2006; Garaj-Vrhovac et al., 2008). It is therefore clear that the CBMN test combining all of the above parameters can give a better insight in different types of cytogenotoxic alterations induced by whole bee venom.

In conclusion, our results indicate that *in vitro* whole bee venom induces cell instability, including its death, dominantly necrosis. In addition, one of the mechanisms of these cell disturbances could be the production of ROS. Since bee venom is a mixture of different active compounds its toxic effects could be largely attributed to its small protein melittin that comprises around 50% of dry venom, phospolipase A_2 which activity is enhanced by melittin and apamin another small peptide that is found in whole bee venom. Those three are the components with known cytotoxic effects towards variety of cells and are likely to be responsible for the effects encountered in our study. Further research about the impact on different types of cell lines may suggest in which direction the application of this natural product should develop in medical practice.

Conflict of interest statement

The authors declare that there have been no conflicts of interest in this research.

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