An influence of cisplatin on the cell culture of *Nicotiana tabacum* BY-2

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ABSTRACT

The mechanism of cisplatin effect has been well examined in *in vitro* models of malignant cell lines, but has never been studied on plant cell lines. When plant cell suspension culture of *Nicotiana tabacum* BY-2 was treated with cisplatin a decrease of viability with its increasing concentration was observed as well as the mitotic index. The structure of nuclei has also been changed in dependence on cisplatin concentration.

Keywords: cisplatin; *Nicotiana tabacum* BY-2; cell viability; nucleus; DNA

Cisplatin or cis-diaminedichloroplatinum is an alkylation agent, whose cytostatic action was proved by Barnett and Rosenberg on bacteria *Escherichia coli*, has an irreplaceable role in treatments of a broad range of malignant disorders despite its substantial undesirable effects. (Kartalou and Essigmann 2001, Boulikas and Vougiouka 2003, Fuertes et al. 2003, Siddik 2003, Wang and Lippard 2005). In *in vitro* systems the strong cytostatic effect was demonstrated, based on binding of cisplatin to nucleophilic centers in DNA, especially to guanine (Pratt and Ruddon 1979, Farah et al. 2000, Chaney et al. 2004). This event results in disturbance of both structure and function of DNA. It was found that cisplatin enters into animal cells by diffusion, inside the cells it converts into its active form – the platinum-water complex; the complex with one molecule of water is the most reactive (Goswami et al. 1989). Cisplatin interacts unspecifically with proteins involved in DNA replication and cell division (Hartley 1985, Farrel 1989). Moreover the pH level in cytoplasm is important (Chaloner 1986). Thanks to these properties cisplatin has a distinctive cytotoxic, mutagenous and carcinogenic effects. Whilst the cisplatin toxicity was proved on many animal cell and tumour cell-lines, no study on plant cell models has been performed so far. In our work we studied the cisplatin influence on plant suspension culture of *Nicotiana tabacum* BY-2 in order to evaluate its cytotoxicity effects on nuclear structure and mitotic indexes of cells.

MATERIAL AND METHODS

Material. The plant cell suspension of tobacco (*Nicotiana tabacum*, line BY-2), originally devel-

Supported by the Grant Agency of the Academy of Sciences of the Czech Republic, Project No. 525/04/P132, and by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. 6215712402 and 1M06030.

This contribution was presented at the 4th Conference on Methods in Experimental Biology held in 2006 in the Horizont Hotel in Šumava Mts., Czech Republic.

oped by Nagata et al. (1992), was cultivated in the Murashige and Skoog medium (Murashige and Skoog 1962), modified by Nagata (Nagata et al. 1992). The cisplatin in the concentrations of 5, 10, 15, 20 and 25 mg/l was added to culture in exponential growth phase. Each concentration was cultivated in five series on a shaker (Kühner Shaker LT-W, Adolf Kühner AG, Switzerland) at 27 ± 1°C and 130 rpm in the dark in 250-ml Erlenmeyer flasks. The samples of the culture were collected in sterile conditions at time intervals of 1, 24, 48, 72, 96 and 120 h.

Chemicals. Cisplatin Ebewe (Ebewe Pharma, Unterach, Austria) was used; for epifluorescence microscopy the fluorescein diacetate (FDA, Sigma Aldrich Chem. Corp., USA) in the form of stock solution in anhydrous acetone, propidium iodide (PI, Sigma Aldrich Chem. Corp., USA) and Hoechst 33342 (Sigma Aldrich Chem. Corp., USA) were used as water stock solution. Other chemicals, such as piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and $MgCl_2$, were obtained from Sigma Aldrich Chem. Corp., USA. Cultivation media were purchased from Duchefa Biochemie BV (Haarlem, Netherlands).

Viability test. The sample of tobacco BY-2 cells was supplemented to the volume of 50 µl and incubated for 5 min at room temperature with FDA (2.4 µmol/l) and PI (30 µmol/l). The percentage of viable and dead cells was determined by counting using the fluorescent microscope (Olympus AX 70) equipped with broad spectrum UV excitation; from each series 5 random fields were evaluated in the microscope and the viability was determined.

The determination of nuclear morphology and mitotic index. For nucleus observation and mitotic index determination, the sample of tobacco cells (4 ml) was treated by adding 4 ml PEM-buffer (100mM PIPES, 10mM EGTA, 10mM $MgCl_2$, pH 6.9) containing formaldehyde (4%, w/w). The sample of fixed cells (50 µl) was incubated for 5 min at room temperature with Hoechst 33342 (4 µmol/l). Nucleolar morphology and mitotic index was determined using the fluorescent microscope (Olympus AX 70) equipped with broad spectrum UV excitation. One thousand nuclei in each preparation were observed.

RESULTS AND DISCUSSION

To determine the cell viability, the fluorescent probe with fluorescein diacetate was used (Guilbault and Kramer 1964). FDA is enzymatic, degradated in living cells by action of plant esterases present in viable cells to green fluorescent fluorescein. Propidium iodide enters to dead cells as a consequence of the disturbance of cell membrane permeabilization and binds into nuclear DNA (Vitecek et al. 2004, Vitecek et al. 2005) (Figure 1). Whereas during the cultivation the viability of untreated tobacco BY-2 cells was well balanced (above 96%), with increasing cisplatin concentration in the cultivation medium the viability decreased gradually (Figure 2). This was noticeable especially after 72 and more hours of cultivation, where the percentage of viable cells was very low (about 5%); after 96 hours no viable cells were observed. The relative standard deviation (referred to as RSD) was smaller than 5%.

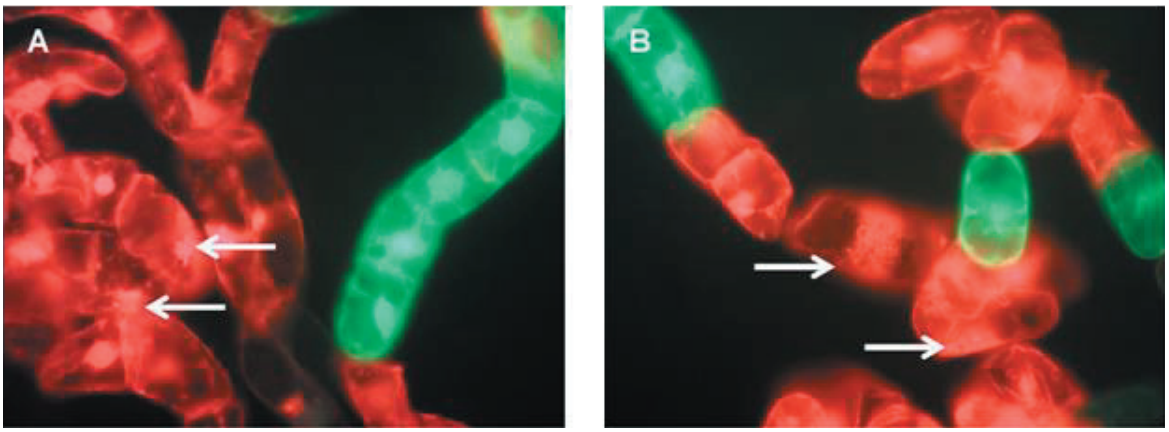

Figure 1. Changes of the BY-2 cells viability and morphology 48 h after cisplatin treatment in the concentration of 10 mg/l. BY-2 cells were stained with FDA and PI and observed using the epifluorescence microscopy. Green cells represent living cells, red cells are lifeless. Arrows show the typical features of programmed cell death – cytoplasm shrinkage and apoptotic-like bodies formation. Magnification is × 400

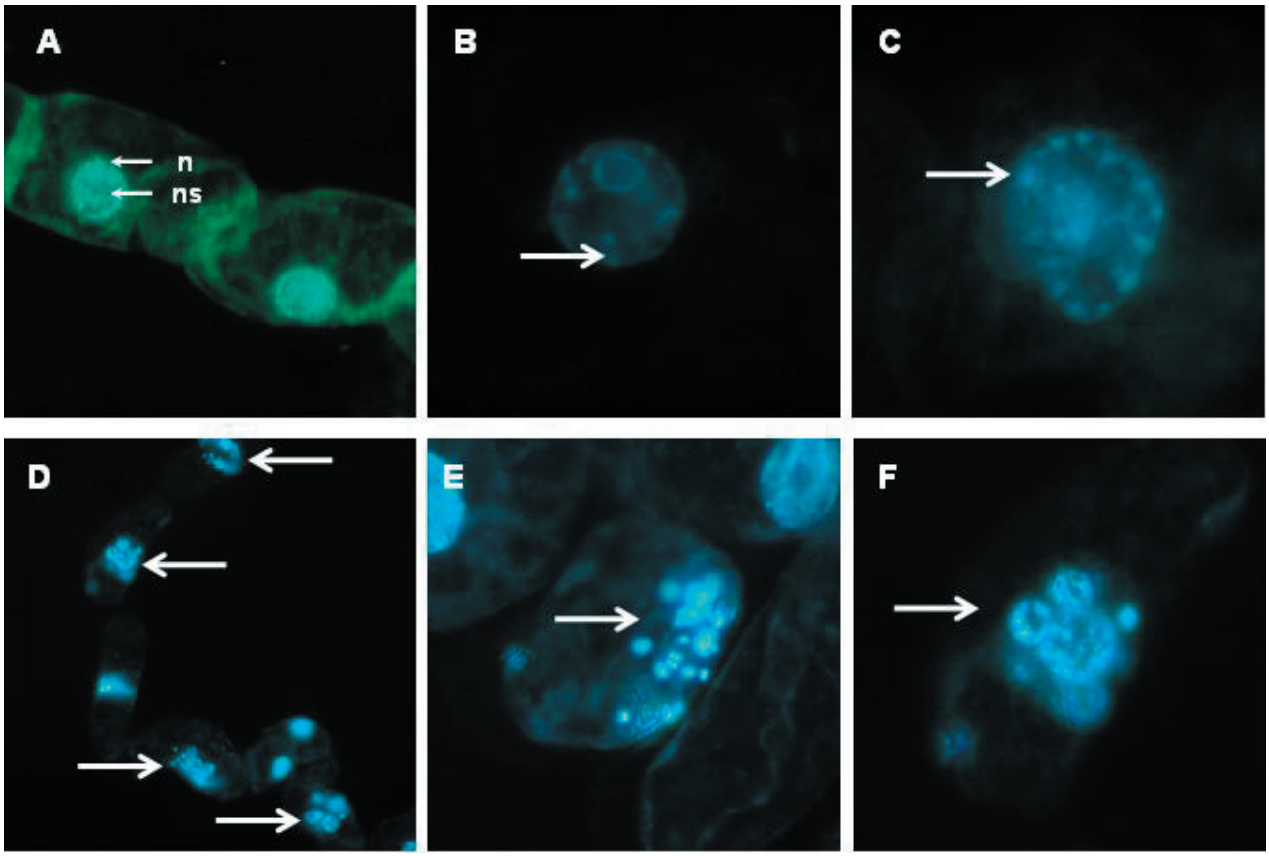

Figure 2. Morphological changes of tobacco nuclei after 48-hour treatment with cisplatin in the concentration of 10 mg/l. Tobacco cell suspension cultures were fixed and stained with Hoechst 33342 and observed with the epifluorescence microscopy. (A) Untreated tobacco cells. (B–F) Tobacco BY-2 cells treated with cisplatin. (B, C) High chromatin condensation at the periphery of nucleus and around nucleoli. (D) Cells showing apoptotic-like nuclei and details. (E, F) Arrows show the typical features. n – nucleus, ns – nucleolus. Magnification is × 400 (A, D) and × 1000 (B, C, E, F)

The viability of BY-2 tobacco cells was already decreased one hour after cisplatin treatment, mainly in the concentration of 20 and 25 mg/l. This observed effect cannot be taken into account in the interaction of cisplatin with DNA, but in its interaction with the reactive biomembrane components and groups of integral membrane proteins, which leads to structural disturbance, permeabilization and subversion of osmotic and ion system of the cell, which results in the cell death. The regression curve of the dependence of cells viability on cisplatin concentration was determined. As shown in Figure 3, the concentration of 15 mg/l for 96 h led to death of practically all BY-2 cells.

Thanks to staining with fluorescent probe Hoechst 33342 we could determine the nuclear morphology and mitotic index of single series of untreated and treated BY-2 cells with RSD less than 3%. The decrease of the mitotic index was very low after one hour after treatment in all concentrations; this, together with the decrease of the viability, confirms the theory of permeabilization of BY-2 tobacco cell biomembranes (Figure 4).

In compliance with the comparison of the structures of nuclei (the structure with well visible nucleolus and fixed chromatin distribution) of untreated control cells and cells treated with low cisplatin concentrations, e.g. to 10 mg/l, we ascertained that the atypical mitosis with unpaired chromosomes can lead to micronuclei formation and the presence of apoptotic bodies with all signs of programmed cell death, such as cytoplasm shrinkage already after 48 h of cultivation (Figure 2). The detected changes give evidence of the ability of cisplatin to enter the cells through the cell barrier of cell wall (by the process of diffusion) at conserving the cell viability and the interaction with DNA inside the cells, which indicates the micronuclei formation.

The apoptotic bodies indicate the ability of cisplatin to start the processes leading to programmed

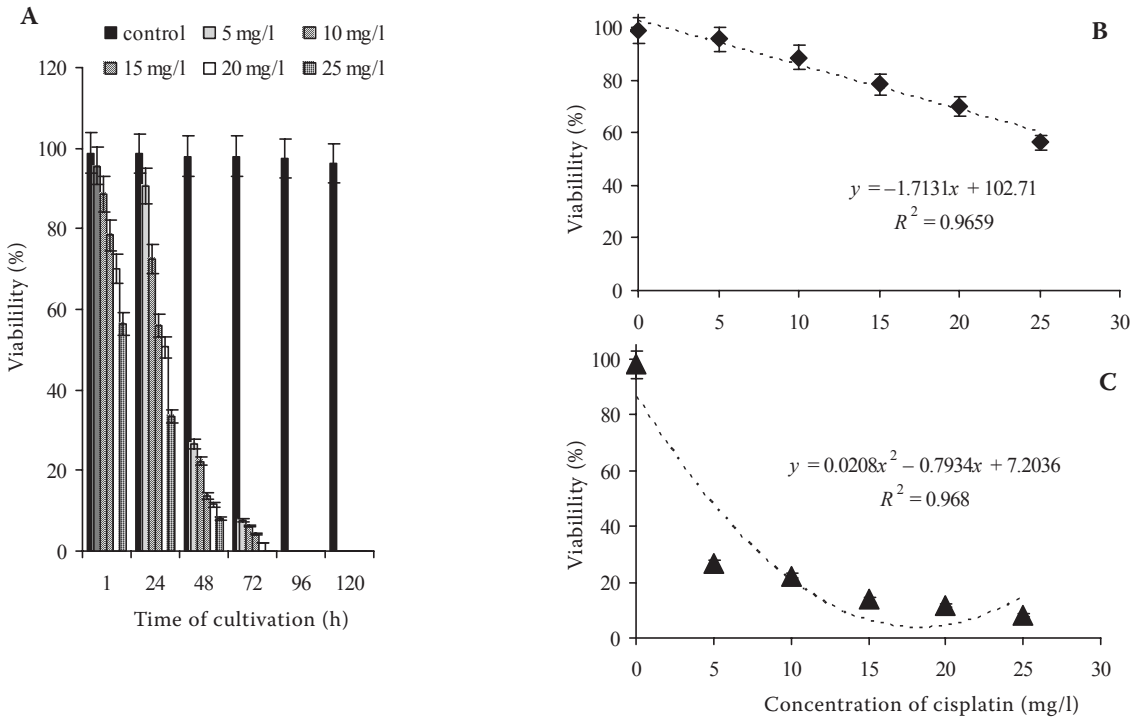

Figure 3. Cultivation time-response graph showing the dependence of various cisplatin concentration treatments on viability of BY-2 cells. Cells were harvested and stained with FDA and PI (A). Dose-response curve showing the effect of cisplatin on tobacco BY-2 cells viability. Cells were harvested 1 h (B) or 48 h (C) after the treatment, stained with FDA and PI and counted. Each point represents three independent experiments

cell death, same as in animal cells, as shown in the works of other authors. Especially at high cisplatin concentration in cultivation medium, significant structural changes of nuclei and nucleoli were observed, namely the vacuolization of nuclei and changes in typical chromatin distribution.

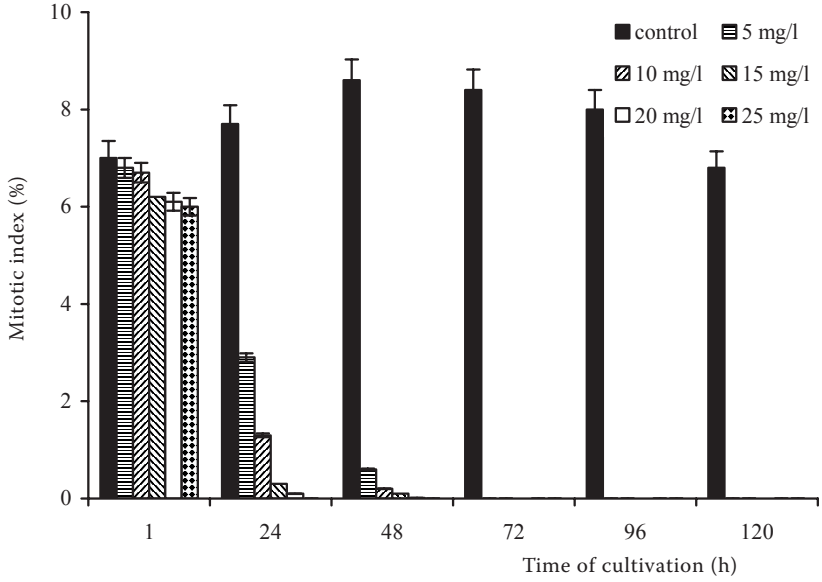

Figure 4. Time-response curve showing the effect of cisplatin on mitotic index of BY-2 cells. Cells were harvested at various times (1, 24, 48, 72, 96 and 120 h) after treatment, stained with Hoechst 33342 and observed using the epifluorescence microscopy. Each point represents three independent experiments

In this work the cytotoxic effect of cisplatin on plant cells of tobacco BY-2 suspension culture was shown, together with markedly decreased mitotic index. The mechanism of this cisplatin action seems to be both disruption of the cell surfaces integrity (cytoplasmatic membrane), where the interaction with reactive function groups of proteins and consequent disturbance of biomembranes permeability can occur, and, in the case of prolonged cultivation, the interaction with DNA and consequent apoptosis induction and atypical mitosis course, which results in micronuclei formation.

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Received on February 26, 2007

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