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DNA damage induced by mutagens in plant and human cell nuclei in acellular comet assay

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Abstract: Higher plant cells have a long tradition of use in the studies on environmental mutagenesis *in situ*, especially in relation to human health risk determination. The studies on the response of plant and human cells to physical and chemical mutagens showed differences in their sensitivity. The differences in the presence of cell components in plants and humans could influence such response. Additionally, the level of the organization of the employed material could influence DNA-damaging effect: leukocytes are isolated cells and plant - an intact organism. To preclude these obstacles, the effects of direct treatment of isolated nuclei with genotoxic agents were determined to compare the sensitivity of plant and human cells. In the present study, we have determined the DNA-damaging effects of two chemical mutagens: maleic acid hydrazide (MH) and N-methyl-N-nitroso-urea (MNU) applied to isolated nuclei of both plant and human cells. In order to compare the sensitivity of the nuclei of *Nicotiana tabacum* var. xanthi and the nuclei of leukocytes, the acellular Comet assay was carried out. The results showed higher sensitivity of the nuclei of leukocytes as compared to the nuclei of plant cells to mutagenic treatment with the applied doses of MH and MNU. (www.cm-uj.krakow.pl/FHC)

Key words: *Nicotiana tabacum* - Leukocytes - Acellular comet assay - DNA damage

Introduction

Since a wide range of genotoxic substances is present in the environment and thus could influence human health, many eukaryotic systems were developed for evaluation of DNA damage. Especially higher plant cells are excellent tools to study environmental mutagenesis *in situ*. The response of plant cells used in bioassays should be as close as possible to that of animal cells, and especially to human ones.

Although a special attention is paid to sensitive *Tradescantia* cells (Trad-SH assay), other plant species are also used as a warning system for the prediction of human health risk. Among many plant systems, the double heterozygous mutant of *Nicotiana tabacum* var. xanthi ($a_1^+/a_1; a_2^+/a_2$) according to its ability to revert phenotypically, observed as the colour changes of single cell in leaf tissue, is unique [8]. The possibility to analyze induced DNA damage, measured as strand breaks in nuclear DNA and point mutation frequency in somatic cells made *Nicotiana tabacum* var. xanthi an excellent model for fundamental

studies, as well as *in situ* environmental monitoring [9, 10, 12, 26].

The comet assay - SCGE (Single Cell Gel Electrophoresis) has become very popular for measuring the level of DNA damage and the effectiveness of repair processes [6]. Its sensitivity and simplicity make it an invaluable tool with widespread application in studies to assess DNA damage and repair in genotoxicity testing, ecological monitoring, as well as human studies [5, 19, 21]. Especially a modified version of this method - the acellular (subcellular) comet assay, in which isolated nuclei are treated by the tested agents, is very useful to determine the effects of direct treatment, and applicable to compare the sensitivity of the cells of different organisms, for example plants and animals. Additionally, the response of intact cells and isolated nuclei can be evaluated, indicating the role of various cellular components in mutagenesis processes [14, 18].

In the study presented here, we have compared the sensitivity of isolated nuclei of plant cells and leukocytes to genotoxic agents using the same genetic endpoints - DNA strand breaks. We used a comet assay on the nuclei of *Nicotiana tabacum* var. xanthi and leukocytes. The leukocytes were applied in this study, as they proved to be alternative cells to lymphocytes, in order to simplify and speed up the comet assay method [4]. DNA-damag-

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ing effect of two chemical mutagens: maleic acid hydrazide (MH) and N-methyl-N-nitroso-urea (MNU), applied to isolated nuclei of plant cells and human leukocytes was determined in the acellular comet assay.

Materials and methods

Chemicals. Mutagens: maleic acid hydrazide (MH; Sigma, CAS 123-3301) and N-methyl-N-nitroso-urea (MNU; Sigma, CAS 684-93-5); agaroses: normal melting point (NMP) and low melting point (LMP) and general laboratory reagents were purchased from Sigma Chemical.

Material. Double heterozygous *Nicotiana tabacum* var. xanthi plants [8] were used in this study. The seeds were kindly provided by dr. T. Gichner (Institute of Experimental Botany, Prague, Czech Republic). Plants were grown in pots to the 5-6 true leaf stage, in the growth chamber at 26°C with continuous light. Leukocytes obtained from a healthy donor were used as a model of human cells.

Isolation of nuclei. Individual leaves of *Nicotiana tabacum* were placed in a small Petri dish containing 200 µl of cold 400 mM Tris-HCl buffer, pH 7.5 (on ice). Using a razor blade, the leaf was gently sliced into a "fringe" to release nuclei into the buffer under yellow light. This method of isolating nuclei proved to be the best in obtaining low values of DNA damage in control cells [9]. Each slide previously coated with 1% NMP agarose and dried, was covered with a mixture of 55 µl of nuclear suspension and 55 µl of LMP agarose (1% prepared with phosphate-buffered saline) at 40°C and cover-slipped. The slide was placed on ice for at least 5 min, and coverslip was removed. Then 110 µl of LMP agarose (0.5%) was placed on the slide and coverslip was mounted again. After 5 min on ice, the coverslip was removed.

Two ml of whole blood collected in heparin-containing vacutainer tube was diluted 1:5 with red blood cells (RBC) lysis buffer (0.15 M NH₄Cl, 12 mM NaHCO₃, 0.16 mM Na₂EDTA, pH 7.0). The mixture was incubated for 10 min at room temperature. After centrifugation (3000 × g, 5 min) the supernatant was decanted. To remove RBC, the isolation step was repeated by adding RBC buffer and centrifugation. After isolation, the leukocyte pellet was suspended in 1.8 ml PBS. The cells were checked for viability in standard way using trypan blue solution.

Slides were previously dipped in 1% NMP agarose, and then the leukocytes mixed with 1% LMP agarose were placed as the first layer (1:1, v/v). Finally, 110 µl of LMP agarose was added as the top layer. The cells were lysed in lysis buffer: 2.5 M NaCl, 10 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, 10% DMSO, pH 10) for 1 h at 4°C in the dark.

Treatment and single cell gel electrophoresis. MH and MNU were freshly dissolved in 400 mM Tris-HCl buffer, pH 7.5 to final concentrations of 0.4 and 4 mM MH and 0.1 and 1 mM MNU. SCGE (Single Cell Gel Electrophoresis) slides with plant or human cell nuclei were treated with the mutagen solutions for 2 h at 26°C, and then they were rinsed three times for 5 min in cold distilled water.

The slides with plant or human cell nuclei were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13) and incubated for 15 min (*N. tabacum* nuclei) and 20 min (leukocyte nuclei). Electrophoresis was performed at 16 V, 300 mA for 30 min (*N. tabacum* nuclei) or 16 V, 300 mA for 20 min (leukocyte nuclei) at 4°C. The electrophoresis conditions used in the study were optimal as they proved to provide low level of DNA damage in control cells and linear concentration-response for the induction of comets after chemical mutagenic treatment in these species in earlier studies [15, 24]. Then the gels were neutralized by washing three times in 400 mM Tris-HCl, pH 7.5 and stained with ethidium bromide (20 µg/ml)

for 5 min. After staining, the gels were dipped in ice-cold distilled water and immediately analyzed.

In each slide, 50 randomly chosen cells were analyzed under the fluorescence microscope with an excitation filter of 546 nm and a barrier filter of 590 nm using computerized image analysis system (Komet Version 3.1. Kinetic Imaging, Liverpool, UK). The tail DNA (TD, %) and tail moment (TM) were used as parameters of DNA damage. The median TD and median TM values were calculated.

Additionally, the visual scoring of comet images was applied. Each of 100 comets per slide was assigned to a category from 0 to 4, according to the degree of damage (0 - undamaged nuclei, 4 - the whole DNA in the tail). The overall score was between 0 and 400 arbitrary units, where the maximum score of 400 indicates that all comets have maximal extension of DNA into the tail [1].

Each experiment was repeated twice and then three slides were analyzed per each experimental group. From the repeated experiments, the average values ±SD of each parameter for each treatment group were calculated from the median values from each slide. The differences between the two groups were statistically evaluated by Student's *t*-test.

Results

DNA damage was observed for the applied concentrations of MH and MNU, both in plant and in human nuclei. The effects of MH and MNU treatment measured as tail DNA and tail moment are illustrated in Figures 1 and 2. The control tail moment value was similar for tobacco and leukocytes - 2.73 and 2.52, respectively. 0.4 mM MH and 0.1 mM MNU induced an increase in DNA damage; nevertheless, the level of DNA damage measured as TM was not significantly different in tobacco and leukocyte nuclei. With lower concentration of MH, the TM values were 8 and 9 for tobacco and leukocytes, respectively. Treatment with 0.1 mM MNU induced DNA damage expressed as TM values: 5.54 for tobacco and slightly, but not significantly higher for leukocytes - 6.28. Treatment with 4 mM MH and 1 mM MNU induced the higher level of DNA damage and resulted in significant differences between tobacco and leukocyte nuclei. The tail moment values of plant cell and leukocyte nuclei treated by 4 mM MH were 12 and 16.5, respectively. The highest level of DNA damage was observed after treatment with 1 mM MNU in plant and human cell nuclei: the respective TM values were 18 and 29. The results of TD analysis also showed that the DNA-damaging effect was higher in the nuclei of leukocytes than in tobacco cell nuclei. The treatment with 0.4 mM MH showed low, but significant differences in TD between leukocyte and tobacco nuclei. After treatment by 1 mM MNU, TD increased from similar value of the control to 32 µm in plant nuclei, and to 44 µm in leukocyte nuclei. The nuclei isolated from plant cells treated with 4 mM MH had lower TD values (27 µm), than leukocyte nuclei (32 µm) exposed to the same mutagen concentration.

The visual evaluation of DNA damage confirmed the results of computer-assisted analysis, which showed higher sensitivity of leukocyte nuclei (Fig. 3). For 4 mM

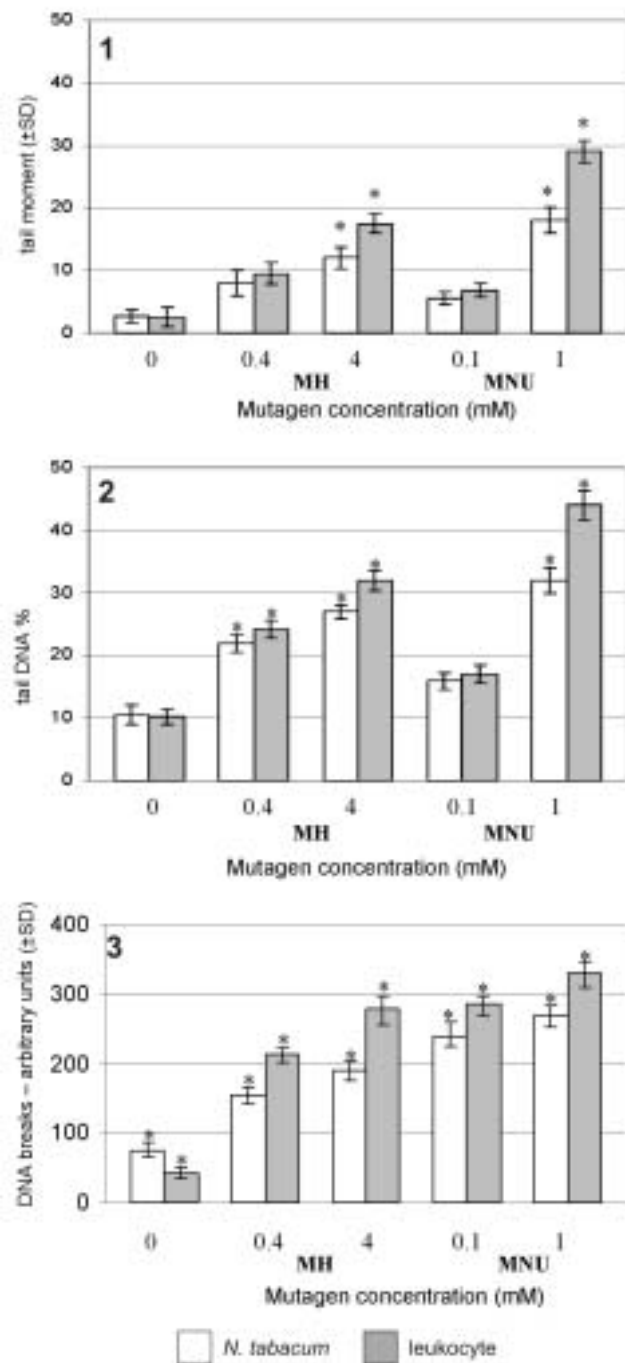


Fig. 1. A comparison of tail moment values (TM) in *N. tabacum* and leukocyte nuclei after treatment with different doses of MH and MNU. *Significantly different ($p < 0.05$) at each dose (t -test). **Fig. 2.** A comparison of tail DNA values (TD) in *N. tabacum* and leukocyte nuclei after treatment with different doses of MH and MNU. *Significantly different ($p < 0.05$) at each dose (t -test). **Fig. 3.** Frequency of DNA breaks measured in arbitrary units by visual scoring of comets in *N. tabacum* and leukocyte nuclei after treatment with different doses of MH and MNU. *Significantly different ($p < 0.05$) at each dose (t -test).

MH, the frequency of DNA breaks, expressed in arbitrary units, was 279 in leukocyte nuclei and 189 in tobacco cell nuclei. These significant differences are emphasized by higher level of DNA damage in control tobacco nuclei than control leukocytes. Similarly, with lower concentration of MH, the nuclei of leukocytes expressed a significantly higher DNA damage than plant cell nuclei. The results of the analysis of DNA damage after MNU treatment also confirmed higher sensitivity of leukocyte nuclei.

To summarize, the results demonstrate higher sensitivity of leukocyte nuclei than plant cell nuclei, especially using higher doses of the applied mutagens. Both mutagens applied to isolated nuclei induced DNA damage, and the strongest effect was observed in the case of 1 mM MNU. The treatment of isolated nuclei by the applied mutagens caused a dose-dependent effect, showed by different parameters of DNA damage.

Discussion

A comparison between genotoxic effects in distinct type cells, of the same or different organisms, which are used in biomonitoring studies, is fundamental to the set-up of the new systems. Among various methods, comet assay is an attractive tool for such comparative studies. SCGE was primarily developed and most commonly applied to animal cells, which do not have the cell wall, which makes isolation of nuclei difficult [22]. Development of the methods for isolation of nuclei from plant tissues have made possible to use SCGE as a test for genetic toxicology and DNA repair in plants [13, 16, 20]. Additionally, comet assay as a simple way of DNA damage measuring allows to compare the genotoxic effects in both plant and animal cell nuclei, including human ones.

The standard (cellular) comet assay, in which the whole organisms, tissues or intact cells are treated with the test agents, allows to study DNA damage, but the cell components and complex organism organization probably influence the response to the treatment. The development of acellular comet assay, in which the nuclei can be treated after embedding in agarose on microscopic slides, offers the possibility to check the effects of genotoxic treatment applied directly to the nuclei. By comparing the data obtained in the cellular and acellular comet assay, the role of the cell wall and the cytosol in the induction of DNA damage by mutagens, as well as the participation of repair enzymes in final effect for tobacco cells was previously determined. Moreover, the application of cellular and acellular comet assay helped to elucidate and compare the mechanisms of action of genotoxic agents: promutagen and direct acting mutagens [14].

In this study, the acellular comet assay enabled, for the first time, the comparison of the effects of DNA-damaging agents applied to human and plant cell nuclei.

There are few studies comparing the sensitivity of plant and human cells, but they in fact did not compare the effects of treatment of nuclei. The study of Poli *et al.* [23] showed, as expected, that the intact plants reveal a weaker response to extracted environmental air-dispersed pollutants measured as the level of DNA damage in comet assay than human leukocytes. It is probably because of multicellular organization of plant body, in contrast to single human blood cells, used for exposition to the tested agents. It has been pointed out earlier that defensive response mechanisms to mutagenic treatment of an intact plant differ from a response of isolated organs or cells as shown in cytogenetic tests [17]. A similar sensitivity of human and plant cells to the same genotoxic agents was reported by Cebulska-Wasilewska [3]. Unfortunately, this conclusion concerns different endpoints of genotoxicity: gene mutations in the Trad-SH assay and cytogenetic damage in human lymphocytes.

The results of this study clearly showed higher sensitivity of the nuclei of leukocytes than of plant cell nuclei to applied mutagens. The differences in sensitivity of the nuclear DNA of human and plant cell nuclei demonstrated in this study could result from the organization of their genomes. Plant and human genomes show striking similarities, but unique differences could influence response to mutagens. The higher sensitivity of human genome to the applied mutagens can be attributed to genome size. Well-known correlation between genome size and the effects of genotoxic treatment could be attributed to our results, taking into consideration differences in the genome size of the investigated species: human - $2C = 3.5$ pg [27] and *Nicotiana tabacum* - $2C = 8.86$ pg [7]. Additionally, the level and the type of DNA damage depend on the cell cycle phase of the treated cells. Nevertheless, leukocytes and nondividing tobacco leaf cells are at similar cell cycle stage - G_0/G_1 , and thus this is not the reason for the differences in their sensitivity. The differences in human and plant chromatin structure, especially the level of chromatin condensation, which reflects heterochromatin/euchromatin proportion, could be responsible for the differences in sensitivity of the compared systems. It is not out of question that higher heterochromatin content in plant genome could reduce the DNA-damaging effect of the mutagenic treatment. The explanation of the differences in the sensitivity of human and plant genome to genotoxic agents could also come from the level of epigenetic DNA modifications, such as specific histone acetylation. Chromatin remodelling may involve removing or repositioning nucleosomes, or simply altering their packing to decondense or recondense the chromatin, which change its accessibility to genotoxic agents. A great deal of evidence concerns consequences of chromatin remodelling for gene expression and nuclear architecture, but still there is no data concerning

the possibility of epigenetic influence on the sensitivity of cells to genotoxic agents [2, 25]. Thus, answering this question, with the help of the acellular comet assay as an excellent tool, is a challenge for the future, which could provide interesting aspects of genetic toxicology.

In this study, the higher sensitivity of human than plant nuclei was demonstrated by two independent methods of comet assessment: by image analysis and by visual examination. Earlier Collins *et al.* [5] have shown a close correlation between results obtained by different DNA damage-measuring methods in human lymphocytes. It is important to note that differences concerning the lysis step in the applied procedure could not influence the results of the present studies. It was confirmed previously that the lysis step in various SCGE experiments with plant material is unnecessary and it does not influence the effects of mutagenic treatment [9].

In the present study, the stronger DNA damaging effect was observed after treatment with MNU than with MH, both in plant cells and leukocytes. Because of the lack of the reservoir of repair system in isolated nuclei, which could change the final effect of the treatment, the difference in kinetics of DNA repair of MH- and MNU-induced damage is not a reason for higher effectivity of MNU treatment. These results could be due only to the differences in their mechanism of action: maleic acid hydrazide (MH) is an S-dependent clastogen, whereas N-methyl-N-nitroso-urea (MNU) is an alkylating agent. Additionally, the results allow to determine if cell components are essential in genotoxic action of the applied mutagens. Our results confirm the earlier ones obtained in the studies with acellular and standard comet assay on murine leukemic cells, which showed that MNU does not require cellular activity to induce DNA damage [18]. Moreover, our results showed that MH also produced comets, when subcellular treatment was used. On the contrary, Gichner *et al.* [11, 15] reported that MH had no significant effect on inducing DNA damage in *Nicotiana tabacum* leaf cells, after the treatment of whole plants. Thus, the differences in sensitivity to MH could come from the way of treatment and thus can be attributed to the complicated mechanisms of a multicellular organism as well as to the presence or absence of cell components.

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