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*Original Research Paper*

# CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY IN HUMAN GLIOMA CELLS EXPOSED TO RADIATION

JERZY SLOWINSKI[1], GRAZYNA BIERZYNSKA-MACYSZYN[2], URSZULA MAZUREK[3], MARIA WIDEL[4], MALGORZATA LATOCHA[3], MONIKA STOMAL[1], MIROSLAW SNIETURA[4] AND RYSZARD MROWKA[1]

[1]Department of Neurosurgery and Neurotraumatology, Medical University of Silesia, Al. Legionow 10, 41-902 Bytom, Poland, [2]Department of Pathology, Medical University of Silesia, Medykow 18, 40-752 Katowice, Poland, [3]Department of Molecular Biology, Biochemistry and Biopharmacy, Medical University of Silesia, Narcyzow 1, 41-206, Sosnowiec, Poland, [4]Department of Radiobiology, Center of Oncology, Wybrzeze Armii Krajowej 15, 44-100 Gliwice, Poland
e-mail: slowian@mp.pl, rmrowka@mp.pl, macyszyn@wp.pl, umazurek@slam.katowice.pl, mlatocha@slam.katowice.pl, widel@io.gliwice.pl
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ABSTRACT

Biological tests are efficient in reflecting the biological influences of several types of generally harmful exposures. The micronucleus assay is widely used in genotoxicity studies or studies on genomic damage in general. We present methodological aspects of cytokinesis-block micronucleus assay performed in human gliomas irradiated *in vitro*. Eight human glioblastoma cell lines obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) were gamma-irradiated ($^{60}$Co) over a dose range of 0-10 Gy. Cytokinesis-block micronucleus assay was performed to quantitate cytogenetic damage. The cells were fixed directly on dishes, stained with fluorochrome DAPI and evaluated under fluorescent and phase contrast microscope. The micronucleus frequency was expressed as a micronuclei (MN) per binucleated cell (BNC) ratio, calculated after scoring at least 100 BNC per dish. The frequency of spontaneous MN ranged from 0.17 to 0.613 (mean: 0.29 ± 0.14). After irradiation increase of MN frequency in the range of 0.312 - 2.241 (mean: 0.98 ± 0.68) was found at 10 Gy. Gliomas are extremely heterogenous in regard to cytogenetic effects of irradiation, as shown in this study by cytokinesis-block micronucleus assay. This test is easily performed on irradiated glioma cell lines and can assist in determining their radiosensitivity. However, in order to obtain reliable and reproducible results, precise criteria for MN scoring must be strictly followed. Simultaneous use of fluorescent and phase contrast equipment improves imaging of morphological details and can further optimize MN scoring.

Keywords: glioma, micronucleus assay, radiosensitivity.

## INTRODUCTION

The cytogenetic assay of micronuclei was established almost thirty years ago (Schmid, 1975). Cytokinesis-block was introduced to micronucleus assay by Fenech and Morley (1985, 1986). Micronuclei (MN) are structures that arise from acentric chromosome fragments or complete chromosomes that failed to attach to mitotic spindle during cytokinesis and are excluded from the daughter nuclei into the cytoplasm (Weissenborn and Streffer, 1992; Fuhrmann *et al*., 1992). MN represent structural chromosomal aberrations (chromosome loss or breakage) induced by ionizing radiation or chemical mutagens (Fenech, 2000). Micronuclei can be measured after DNA staining by the Feulgen reaction, Giemsa or fluorescence dyes. Evaluation of micronucleus frequency is widely used for determination of cellular radiosensitivity (Wandl *et al*., 1989; Shibamoto *et al.,* 1991, Widel *et al*., 2001; 2003). Generally, radiosensitive cells are more susceptible to radiation-induced micronucleus formation. In the previous study, performed by one of the authors (MW), usefulness of cytokinesis-block micronucleus (CBMN) assay in evaluating radiosensitivity of malignant melanoma was proved (Widel *et al*., 1997). Human gliomas, like melanomas, are very heterogeneous tumours in regard to their response to radiotherapy. This prompted us to validate applicability of CBMN assay in irradiated human glioma cell lines and to discuss some methodological aspects related to scoring MN *in vitro*. The test may allow the grouping of gliomas in terms of sensitivity to

radiation and to identify tumours with favourable response after radiation treatment.

# MATERIALS AND METHODS

## CELL CULTURE AND IRRADIATION

Eight human glioma cell lines (42-MG-BA, DBTRG-05-MG, DK-MG, GAMG, GOS-3, LN-405, 8-MG-BA and U-138-MG) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) (Drexler *et al*., 2001). Cells were cultured as monolayers in Easy Y Flasks (Nunc) in DMEM or RPMI medium (according to the manufacturer's recommendations), supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C, in a humid atmosphere of 5% $CO_2$/95% air. The cultures were trypsinized and plated (3-5 × $10^5$ cells per plate) into 50 mm plastic culture dishes (Nunc) in 5 ml of growth medium. After 24 hrs (time required for cell attachment) the cells were irradiated in culture dishes using Philips $^{60}$Co γ-ray unit at a dose rate 0.8 Gy/min, at room temperature to a total dose of 0, 2, 4, 6, 8 and 10 Gy. The control (non-irradiated) cells were sham exposed for the same period. For subsequent irradiation at a given dose each culture was plated into three dishes, so the data of individual cell lines were pooled as triplicates.

## MICRONUCLEUS ASSAY

CBMN assay with cytokinesis-block induced by cytochalasin B was performed according to the method described by Shibamoto *et al*. (1991) and Fuhrmann *et al*. (1992), with minor modifications. Cytochalasin B is used to inhibit the cell division after mitosis. This leads to cells which contain two nuclei, and micronuclei, if chromosome breaks have occurred or the centromere is damaged. Immediately after irradiation the cells were treated with cytochalasin B (Sigma), dissolved in dimethylosulfoxide to a final concentration of 2 μg/ml. After 72 h incubation the cells were fixed in cold (-20ºC) ethanol 96º for 15 min, washed with distilled water, re-fixed in acetic acid:methanol (1:3 v/v) for 15 min and washed. The fixed cells were stained with a drop of fluorochrome DAPI (4',6'-diamidino-2-phenylindole HCl, Serva), dissolved in Tris-buffer (pH 7.5) at a 2 μg/ml concentration. The control (non-irradiated) cells were also incubated with cytochalasin B.

## MICROSCOPIC EVALUATION AND MN SCORING

The preparations were evaluated under photo-microscope Axiophot (Zeiss, Germany), equipped with fluorescence illuminator (HBO50 lamp) and the filter set for 365 nm excitation and 420 nm emission wave length (specific for fluorochrome DAPI). The phase contrast equipment was also used. Analysis was performed at magnification ×400. To facilitate discrimination of MN and differentiation between mononucleated, binucleated (which divided once after irradiation) and polynucleated cells (which divided twice or more), the phase contrast was used together with fluorescent microscopy. Microscopic analysis was performed by one observer (MW), excluding the possibility of determining inter-observer variability, where a series of preparations was viewed by a second experienced observer. Scoring was performed blindly. The percentage of binucleated cells (%BNC) per dish was evaluated after scoring 1000 consecutive cells distributed as a monolayer. The BNC were selected according to criteria described by Fenech *et al*. (2003). The apoptotic and necrotic cells were included in the total cell number, however micronuclei were not scored in these cells. The number of MN in at least 100 BNC was scored according to the criteria of Countryman and Heddle (1976), Fenech and Morley (1985), Falkvoll (1990) and Fenech *et al*. (2003), and the MN/BNC ratio was calculated. The round or oval, non-refractile bodies detached from the nucleus, smooth-outlined and with a diameter not greater than one-third the large diameter of the main nuclei were considered as MN. Only MN separated from the main nucleus and just touching but not overlapping the main nucleus were scored. The experimental data presented in Table 1 and Fig. 1 are the mean values from 3 independent dishes per experimental point. The MN frequency in control (non-irradiated) cells, representing so-called spontaneous MN was regarded as a baseline level. The percentage of binucleated cells (%BNC) in non-irradiated cells was used for measurement of proliferation fraction *in vitro* (Widel *et al*., 2001).

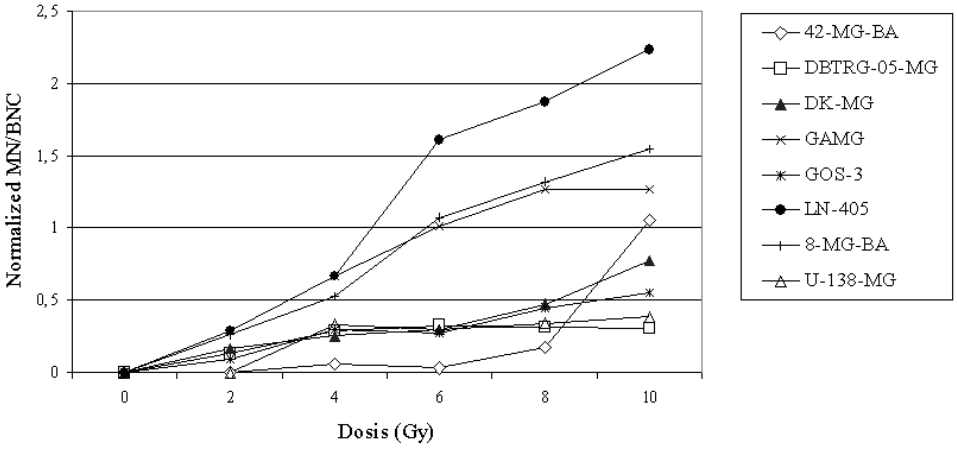


Fig. 1. *Normalized micronuclei per binucleated cell (MN/BNC) ratio in irradiated human glioma cell lines. A dose dependent increase in micronuclei frequency and relative radioresistance of most cell lines is seen.*

Table 1. *Percentage of binucleated cells and absolute values of micronuclei per binucleated cell ratio in eight human glioma cell lines after gamma-irradiation.*

| Cell line | %BNC | | | | | | MN/BNC | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0Gy | 2Gy | 4Gy | 6Gy | 8Gy | 10Gy | 0Gy | 2Gy | 4Gy | 6Gy | 8Gy | 10Gy |
| LN-405 | 48.54 | 49.79 | 47.02 | 44.59 | 41.8 | 27.76 | 0.30 | 0.59 | 0.97 | 1.91 | 2.17 | 2.54 |
| 8-MG-BA | 62.5 | 61.45 | 59.11 | 53.81 | 48.36 | 37.17 | 0.18 | 0.45 | 0.71 | 1.26 | 1.51 | 1.73 |
| GAMG | 49.51 | 36.49 | 34.45 | 30 | 32.15 | 24.54 | 0.17 | 0.46 | 0.84 | 1.18 | 1.44 | 1.44 |
| DK-MG | 17.53 | 15.83 | 15.31 | 15.3 | 15.43 | 13.6 | 0.61 | 0.78 | 0.87 | 0.91 | 1.09 | 1.39 |
| DBTRG-05-MG | 8.13 | 8.42 | 8.15 | 8.57 | 7.6 | 7.7 | 0.17 | 0.30 | 0.46 | 0.50 | 0.49 | 0.48 |
| GOS-3 | 19.03 | 15.96 | 9.63 | 9.47 | 8.37 | 9.3 | 0.25 | 0.34 | 0.54 | 0.52 | 0.70 | 0.80 |
| 42-MG-BA | 13.8 | 14.3 | 13.41 | 12.07 | 10.81 | 11.16 | 0.36 | 0.31 | 0.42 | 0.39 | 0.53 | 1.41 |
| U-138-MG | 28.76 | 26.45 | 26.93 | 19.53 | 16.25 | 13.31 | 0.36 | 0.36 | 0.70 | 0.66 | 0.70 | 0.75 |

%BNC: percentage of binucleated cells; MN/BNC: absolute values of micronuclei per binucleated cell ratio

## STATISTICAL ANALYSIS

The correlation between radiosensitivity of cell lines (expressed as a normalized MN/BNC ratio) and the proliferation fraction (expressed as a %BNC in non-irradiated controls) was evaluated using the nonparametric Spearman's rank correlation coefficient. Variation of the MN/BNC ratio measurements was measured with the coefficient of variation (CV):

$$CV = \frac{SD}{\overline{x}} \times 100\% \qquad (1)$$

SD: standard deviation; $\overline{x}$ : the mean

Inter- and intra-tumour, as well as inter- and intra-observer variations were estimated using one-way ANOVA test and Spearman's rank correlation coefficient. The means were compared with U Mann-Whitney test. The level of significance was set to $P < 0.05$.

## RESULTS

The MN/BNC ratio in non-irradiated and irradiated cells is presented in Table 1. For better visualization of percentage increment in MN frequency after irradiation the baseline values were subtracted (normalized MN/BNC) (Fig. 1). The CBMN assay with DAPI staining provided satisfactory preparations, enabling reliable identification and scoring of MN (Fig. 2).

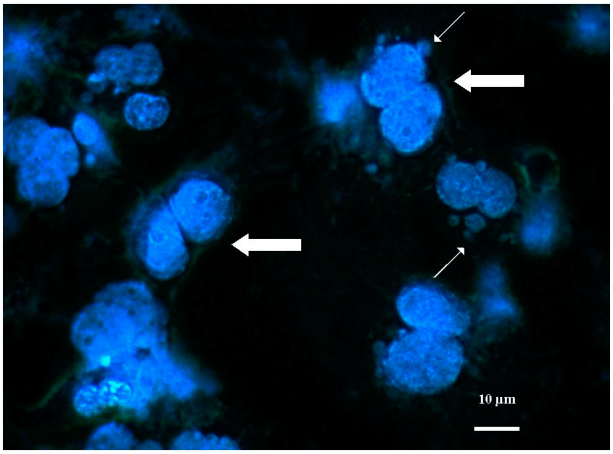


Fig. 2. 8-*MG-BA cell line after 10 Gy exposure. Numerous binucleated cells (large arrows) containing multiple micronuclei (small arrows). The micronuclei have a similar structure and intensity to the main nuclei. DAPI stain. Fluorescent microscopy. Original magn. ×200.*

The examined glioma cell lines showed high heterogeneity in respect to genotoxic effects of ionizing radiation, as quantified with CBMN assay. Generally, a dose-dependent increase of MN frequency was observed. The majority of cell lines, excluding LN-405, 8-MG-BA and GAMG (arbitrarily labeled radiosensitive) proved to be highly radioresistant (Fig. 1).

The number of spontaneous MN/BNC ranged from 0.17 to 0.613 (mean: 0.30 ± 0.15). After irradiation the increment of MN/BNC ratio (*i.e.*, normalized MN/BNC) in the range of 0.312 - 2.241 (mean: 0.98 ± 0.68) was seen at a dose of 10 Gy. A synchronous dose-dependent decrease in % BNC was seen due to radiation-induced cell cycle delay (Table 1). However, there was a high correlation between %BNC in non-irradiated controls and MN/BNC ratio in irradiated cells (Spearman's R range: 0.55 - 0.85, significant at 6 Gy (P = 0.010) and 8 Gy (P = 0.007)) (Fig. 3). The radiosensitive and radioresistant lines were discriminated by each normalized MN/BNC and %BNC value as well as by absolute MN/BNC values after 6, 8 and 10 Gy doses (p = 0.025). The coefficient of variation of three consecutive MN/BNC values obtained in a single cell culture at a given irradiation dose ranged from 11.32% to 23.91% (mean: 15.48%). For comparison, CV of scorings performed at a given dose in a total of 8 cell lines ranged from 37.8% to 68.6% (mean: 54.95%). The intra-tumour and inter-tumour mean CV differed significantly (P < 0.0001, one-way ANOVA). In GOS-3 glioma cell line the MN/BNC ratio was scored independently by two observers. There was no significant difference between the two series of measurements (P = 0.97, one-way ANOVA) (Fig. 4). The mean MN/BNC in 18 consecutive measurements performed in this culture by the two observers was nearly identical (0.525 vs. 0.530).

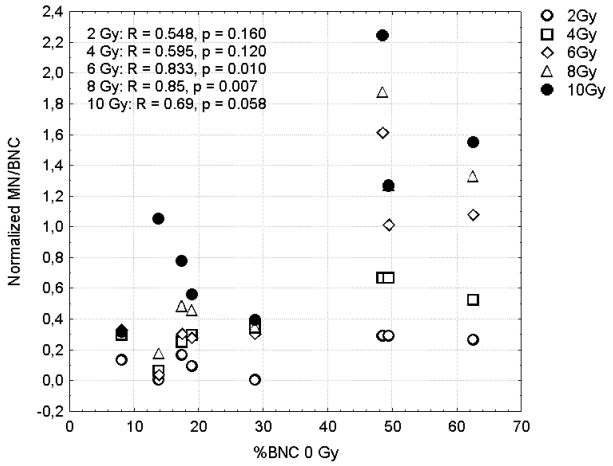


Fig. 3. *A scatterplot of normalized micronucleus frequency (MN/BNC) after 2, 4, 6, 8, and 10 Gy irradiation vs percentage of binucleated cells (%BNC) in non irradiated controls.*

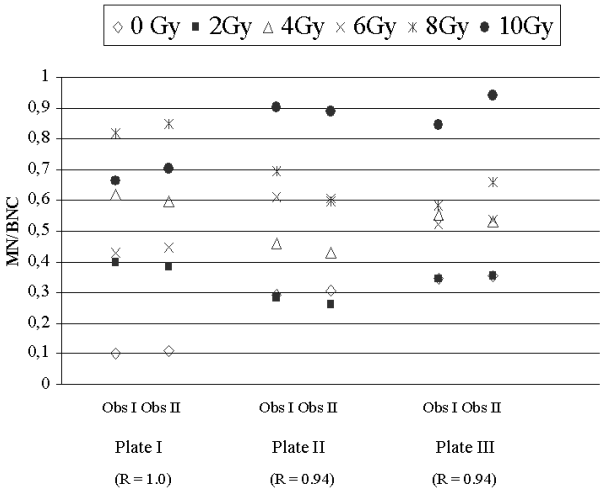


Fig. 4. *A scatterplot of absolute values of micronucleus frequencies (MN/BNC) scored by two observers (Obs I and Obs II) on three consecutive plates in GOS-3 line. R - Spearman's coefficient of correlation. High inter-observer reproducibility is confirmed.*

## DISCUSSION

In the present study, the background and radiation-induced DNA damage was measured with the micronucleus assay. Cytokinesis block used in the assay enables accurate evaluation of MN generation after exposure, since cells undergoing mitosis at the time of irradiation (*i.e.*, cells potentially generating micronuclei) are easily identified as binucleated cells (Shibamoto *et al*., 1991; Fuhrmann *et al*., 1992; Fenech, 2000). The main advantages of CBMN assay are relative rapidity and simplicity, especially as compared with clonogenic assay, measuring the ability of tumour cells to produce a colony of descendants (Steel, 1993; Widel *et al*., 1997). The micronucleus assay does not require the clonogenicity of cells.

In the analysis of intra-tumour and inter-tumour variability of MN/BNC the estimated CV differed significantly. This finding confirms applicability of CBMN assay for measurement of cellular radio-sensitivity in glioma cells *in vitro*. In our opinion following the strict criteria for scoring of MN guarantees high reproducibility of the results (inter-observer variation). Simultaneous use of phase contrast microscopy enables more accurate definition of cell borders, reducing the risk of MN misinter-pretation (Fig. 5).

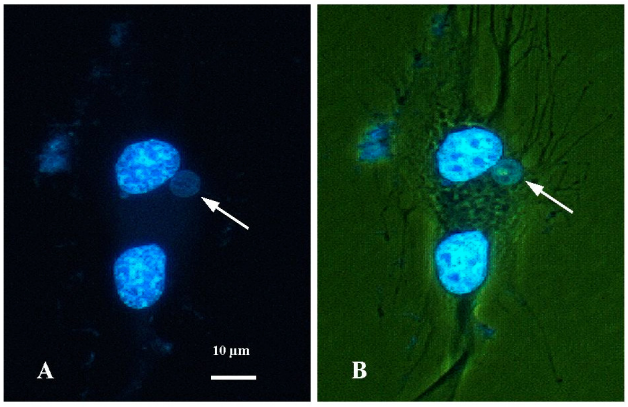


Fig. 5. *GOS-3 cell line after 6 Gy exposure. Large micronucleus is seen within binucleated cell (arrow). Binucleation, not clear in fluorescent microscope (A) becomes evident after application of phase contrast equipment (B). DAPI stain. Fluorescent - phase contrast microscopy.*

Automated scoring of MN by computerized image analysis systems is an interesting alternative to tedious manual scoring. It gives results comparable to those obtained by manual scoring (Castelain *et al*., 1993, Verhaegen *et al*., 1994) with even better reproducibility (Thierens *et al*., 1997). However, automated MN scoring is time-consuming and is thus applicable only to a limited number of slides (Thierens *et al*., 1997). The studies on automated MN scoring were mostly performed on normal lymphocytes (Castelain *et al*., 1993; Szirmai *et al*., 1993, Verhaegen *et al*., 1994), *i.e.*, cells with relatively uniform morphology as compared to highly pleomorphic cells of human gliomas. Probably, the developing of an appropriate algorithm for automated MN scoring in cultured gliomas would be a very demanding task. Flow cytometric analysis was also used for MN scoring. The main disadvantage of this approach is that apoptotic cells and apoptotic bodies, if present, can influence MN measurement (Nüsse and Marx, 1997). It would probably be a significant obstacle in analysis of irradiated gliomas, as in the studied cell lines the apoptotic index reached over 10% in 4 of 8 lines (data not shown).

Analysis of the slides prepared for MN scoring provides additional data regarding cellular kinetics and survival. For example, %BNC and nuclear division index, based on the ratio of mononucleated, bi- and multinucleated cells reflect proliferative activity (Keshava *et al*., 1996; Widel *et al*., 1997; Fenech *et al*., 2003; Palyvoda *et al*., 2003), (Fig. 6). In our study we found a high correlation between %BNC and normalized MN/BNC ratio that suggests greater radiosensitivity in rapidly proliferating cells. The correlation between %BNC and MN frequency could also be explained by a lower apoptotic rate in rapidly dividing cells. However, the mean apoptotic index in rapidly proliferating lines (LN-405, 8-MG-BA, GAMG) was not significantly lower than in other lines (data not shown).

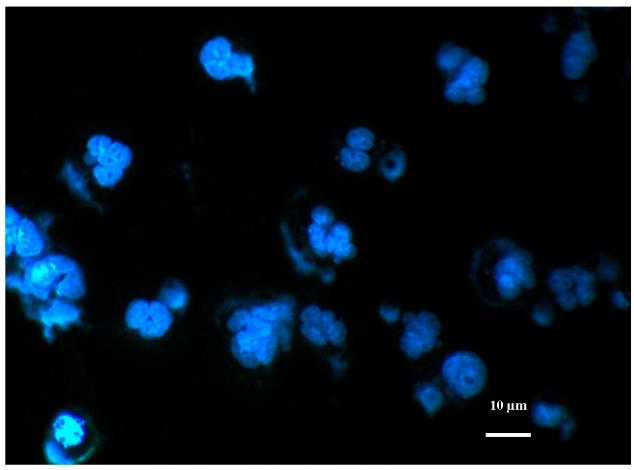


Fig. 6. *LN-405 cell line after 2 Gy exposure. Numerous multinucleated cells suggest high proliferative activity (nuclear division index). DAPI stain. Fluorescent microscopy.*

The nucleoplasmic bridge frequency in BNC can be used as a biomarker of chromosome rearrangement (Fenech, 2000). Furthermore, apoptotic index can be measured on the same slides as well (Falkvoll, 1990; Abend *et al*., 2000) (Fig. 7).

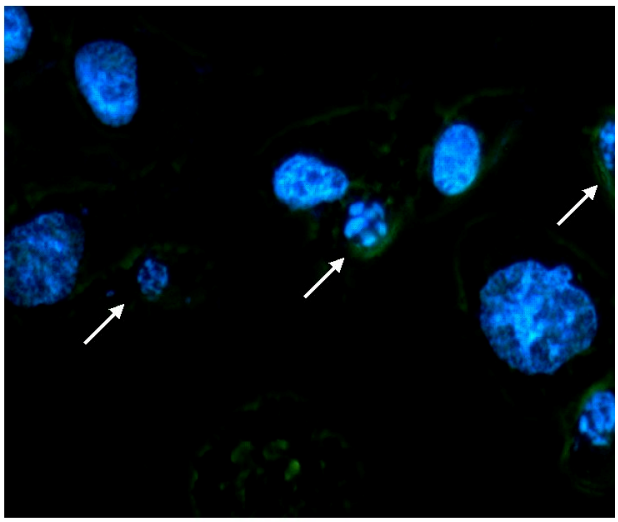

Fig. 7. *DK-MG cell line after 10 Gy exposure. Numerous mononucleated apoptotic cells showing condensed chromatin and early nuclear fragmentation (arrows). DAPI stain. Fluorescent - phase contrast microscopy.*

It is assumed that frequency of MN is a reliable marker of cellular radiosensitivity (Wandl *et al*., 1989; Shibamoto *et al*., 1991; Mariya *et al*., 1997), although some authors do not agree with this assumption (Bush and McMillan, 1993; Akudugu *et al*., 2000). Generally, increase in micronucleus frequency after irradiation is indicative of better response to radiotherapy (Zölzer *et al*., 1995). MN assay is also used for retrospective dosimetry after exposure to ionizing radiation (Streffer *et al*., 1998), for biological monitoring of populations exposed to mutagenic and carcinogenic agents (Fenech, 2000) and for quantification of DNA damage/repair processes (Palyvoda *et al*., 2003).

There are many potential research applications of the described test. For example, we have used the CBMN assay for evaluating radioprotective properties of histone proteins and the relation between cellular proliferation (evaluated by *in situ* hybridization for histone mRNA) and radioresistance (Slowinski *et al*., 2003a,b).

## ACKNOWLEDGEMENTS

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