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EVALUATION OF *IN VITRO* GENOTOXIC ACTIVITY OF BLEOMYCIN AND MITOMYCIN C IN HUMAN LYMPHOCYTES USING THE ALKALINE COMET ASSAY*

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Although chemotherapy targets cancer tissue, it also damages the DNA of non-cancer cells. The aim of this study was to evaluate the *in vitro* genotoxic potential of therapeutic concentrations of bleomycin and mitomycin C, added alone or in combination to cultures of human peripheral lymphocytes. The levels of DNA damage and repair were assessed using the alkaline comet assay immediately after cell treatment as well as 24 and 48 hours following treatment. The results indicate that individual drugs and their combination induce a significant DNA damage to peripheral blood lymphocytes. Bleomycin alone induced the highest levels of primary DNA damage immediately after cell treatment. Although mitomycin C alone induced massive cross-linking and retarded DNA migration in resting cells, active proliferation and repair processes significantly increased DNA damage. Combined, they showed a synergetic effect, inducing complex patterns of DNA damage in peripheral blood lymphocytes and producing different types of lesions and a number of DNA alterations that directly or indirectly increased DNA migration. Our study has confirmed the sensitivity of the alkaline comet assay for assessing bleomycin and/or mitomycin C genotoxicity to human lymphocytes at concentration levels used in clinic. It has also demonstrated the utility of the alkaline comet assay as one of the primary screening methods for *in vitro* studies of drug-DNA interactions, especially in studying mechanisms of action of new drugs.

KEY WORDS: *antineoplastic drugs, chemotherapy, DNA damage, DNA repair*

Cytotoxic drugs are a unique therapeutic class of fundamental importance in current antineoplastic chemotherapy. They belong to different chemical and chemotherapeutic classes. Many of them are genotoxic, carcinogenic and teratogenic when tested *in vivo* and *in vitro* (1, 2). Patients suffering from disseminated malignant diseases are often treated with a combination of antineoplastic drugs which have considerable side effects in cell lines expressing higher growth and replication rates. Mutagenic treatment of a primary cancer may further increase the risk of the development of a secondary cancer, as it induces heritable alterations of tumour-associated genes. Different studies have shown that patients in successful remission have a higher risk for secondary malignancies, including leukaemia and solid tumours. Their incidence correlates with the type of treatment patients received; those experiencing the most intensive treatment run the highest risk (3).

Bleomycin and mitomycin C are potent and widely used antineoplastic antibiotics. Bleomycin is used either in monotherapy or in combination therapy to treat several malignancies including soft tissue carcinomas, squamous cell carcinoma, testicular tumours, non-Hodgkin's lymphoma, and Kaposi sarcoma (4-8). It is described as a radiomimetic

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agent capable of inducing single- and double-strand breaks in DNA - with consequent chromosome aberrations - in all phases of the cell cycle (9, 10). Cells in the M and G_2 phase are the most sensitive to bleomycin toxicity (11). It induces a wide spectrum of mutagenic lesions in mammalian cells, including DNA base damage and apurinic/apyrimidinic sites and is also capable of cleaving RNA (12-14). Mitomycin C is an important component in combined chemotherapy of breast, lung, and prostate cancer, and is among the few drugs effective against colorectal cancer and superficial bladder cancer (15). It also produces many biological effects in mammalian cells or microorganisms: selective inhibition of DNA synthesis, recombination, chromosome breakage, induction of sister chromatid exchanges and DNA repair (SOS response) in bacteria (16-22). Mitomycin C mainly alkylates the N-2 position of guanine, upon reductive activation cascade, to form either monoadducts or intra- or interstrand cross-links (23). Since interstrand cross-links prevent DNA strand separation and can completely block DNA replication and transcription, the toxicity of mitomycin C has mainly been correlated with the formation of these adducts (15).

Despite extensive research, the mechanisms of action of bleomycin and mitomycin C are still controversial and the molecular basis of their genotoxicity has yet to be understood. Although a precise definition of "genotoxicity" is elusive, there is no doubt that DNA damage plays an important role in most mechanisms underlying the action of anticancer drugs interacting with DNA. It is therefore an imperative task in chemotherapy to determine the DNA-damaging effect of these drugs in normal cells (24). Because of their accessibility, human peripheral lymphocytes are among the most frequently studied "normal" human cells. They are often used as an indicator of DNA damage and are most commonly tested using the comet assay (25). This study used lymphocyte cultures as a model system because their growth patterns are similar to those observed in malignant tumours, where not all cells are in the same phase of the cell cycle.

The aim of our study was to evaluate *in vitro* the genotoxicity of bleomycin and mitomycin C, alone or combined using the alkaline comet assay as a simple method for the assessment of DNA damage and repair. This method has been well documented to allow sensitive detection of primary DNA lesions and measurement of repair kinetics at the level of single cells (25-29).

MATERIAL AND METHODS

Blood sampling

Peripheral blood was obtained from a healthy male donor (age 22 years, non-smoker). The donor had not been exposed to ionizing or non-ionizing radiation for diagnostic or therapeutic purposes and to known genotoxic chemicals for a year sampling. The blood sample (V=20 mL) was collected by venipuncture into heparinised tubes (BD vacutainer, Becton Dickinson, N.J., USA).

Experiments *in vitro*

Antineoplastic drugs

Stock solutions of bleomycin (Bleocin, Nippon Kayaku Co., Ltd) and mitomycin C (Mitomycin C, Sigma) were prepared in sterile bi-distilled water. Final concentrations in culture were 0.39 μg/mL for bleomycin and 0.31 μg/mL for mitomycin C. These doses are within the therapeutic range (15 mg/m^2 for bleomycin and 12 mg/m^2 for mitomycin C).

Cell cultures

Lymphocyte cultures were established from freshly drawn heparinized blood. Whole blood (V=0.8 mL) was added to 8 mL of F-10 tissue culture medium (Gibco) containing 20 % foetal bovine serum (Sigma) supplemented with penicillin and streptomycin (Pliva). The cultures were stimulated with phytohaemagglutinin (PHA, Murex; V=0.2 mL).

Bleomycin and mitomycin C treatment

Two different exposure conditions and treatments were designed, as follows:

Treatment A (on G_0 resting cells that were then induced to start the cell cycle). Appropriate volumes of bleomycin, mitomycin C and their combination were added to lymphocyte cultures at the moment of the culture set up. The exposure lasted 24 hours.

Treatment B (on proliferating lymphocytes). Antineoplastic drugs were added *in vitro* to lymphocyte cultures that had been grown for 24 hours. The exposure lasted 24 hours.

After 24-hours of exposure, media containing antineoplastic drugs were carefully removed. Lymphocytes were re-suspended in fresh tissue culture media and cultivated *in vitro* for another 48 hours.

In both experiments one culture was left untreated for negative control (i.e. no drug was added). Control culturing followed the same procedure as all other cultures (with regard to the replacement of fresh medium).

DNA damage and repair were evaluated using the alkaline comet assay immediately after the treatment, as well as 24 and 48 hours after the treatment. For this purpose, aliquots of 10 μL lymphocyte suspensions were taken and microgels were prepared in sterile conditions.

The alkaline comet assay

The comet assay was carried out under alkaline conditions, as described by *Singh et al.* (30). Two parallel replicate slides per sample were analysed. Agarose gels were prepared on fully frosted slides coated with 1 % and 0.6 % normal melting point (NMP) agarose (Sigma). Lymphocyte samples (5 μL) were mixed with 0.5 % low melting point (LMP) agarose (Sigma), placed on the slides and covered with a layer of 0.5 % LMP agarose. The slides were immersed for 1 h in freshly prepared ice-cold lysis solution [2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris-HCl, 1 % Na-sarcosinate (Sigma), pH 10] with 1 % Triton X-100 (Sigma) and 10 % dimethyl sulfoxide (Kemika). Denaturation and electrophoresis were carried out at 4 °C under dim light in freshly prepared electrophoretic buffer (300 mmol/L NaOH, 1 mmol/L Na_2EDTA, pH 13.0). After 20 min of denaturation, the slides were randomly placed side by side in the horizontal gel-electrophoresis tank, facing the anode. Electrophoresis at 25 V (300 mA) lasted another 20 min. The slides were then gently washed with a neutralisation buffer (0.4 mol/L Tris-HCl, pH 7.5) three times at five-minute intervals. Ethidium bromide (20 μg/mL) was used for staining and the slides were stored at 4 °C in humidified sealed containers until analysis.

Comet capture and analysis

Each slide was examined using a 250x magnification fluorescence microscope (Zeiss, Germany) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. A total of 100 comets per sample were scored (50 from each of two replicate slides). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. Using a black and white camera, the microscope image was transferred to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.). To avoid the variability, one well-trained scorer scored all comets. Three parameters of DNA damage were analysed: tail length (presented in micrometres), tail DNA (%) and tail moment.

Statistical analysis

Statistical analyses were carried out using Statistica 5.0 for Windows (StatSoft, Tulsa, USA). The extent of DNA damage was analysed considering the mean (± standard error of the mean), median, range and dispersion coefficient (H) of the comet tail length. Dispersion coefficient was calculated as the ratio between sample variance and sample mean to determine the effect of exposure on the distribution of comet tail lengths within each sample. Cells were also classified as either "undamaged" or "damaged" with respect to threshold levels indicating comets with a long-tailed nucleus (LTN), i.e. the length over the 95th percentile of the distribution of the tail parameters among control samples (31). Multiple comparisons between groups were done using multifactor ANOVA. In order to normalise distribution and to equalize variances, data were log-transformed [$x' = \log(x+1)$]. Post-hoc analysis of differences was done using the Scheffé test. The level of statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

The results of this study are summarised in Tables 1 (treatment A) and 2 (treatment B), showing the mean values and medians for each sample analysed. As the comet measurements were not normally distributed, the results are also presented as the frequency distributions of comet tail lengths derived from the measurement of 100 nuclei per sample. Histograms showing the frequency distribution of comets with regard to their tail lengths are shown in Figures 1 (treatment A) and 2 (treatment B). Figure 3 shows comets in treated and control samples.

The alkaline comet assay detected even subtle differences in DNA migration in normal untreated lymphocytes. The results shown in Tables 1 and 2 and in Figures 1 and 2 indicate that untreated lymphocytes exhibited different levels of primary DNA damage, depending on their position in the cell cycle. These results are in accordance with data obtained

Table 1 *Results of the alkaline comet assay on control peripheral blood lymphocytes and lymphocytes treated with bleomycin (0.39 μg/mL), mitomycin C (0.31 μg/mL) and their combination* in vitro *for 24 hours. Antineoplastic drugs were added to the cell cultures at the moment of culture initiation (treatment A).*

	TAIL LENGTH / μm						TAIL DNA %				TAIL MOMENT				ΣA /100 cells
	Min.	Max.	Mean ±S.E.	Median	H	LTN	Min.	Max.	Mean ±S.E.	Median	Min.	Max.	Mean ±S.E.	Median	
Bleomycin															
1	15.38	97.43	36.47±1.80[a]	30.13	8.99	83	<0.01	28.45	7.07±0.74[b]	4.30	<0.01	7.04	1.34±0.15[a]	0.83	-
2	13.46	96.79	25.45±1.34[b]	20.83	7.09	59	<0.01	19.20	4.64±0.51[b]	2.81	<0.01	8.11	0.79±0.12[b]	0.42	4
3	13.46	41.02	20.56±0.48	19.23	1.10	65	<0.01	10.79	1.35±0.22	0.46	<0.01	1.88	0.22± 0.04	0.07	2
Mitomycin C															
1	13.46	50.00	21.61±0.62	19.87	1.77	31	<0.01	29.47	4.35±0.51[b]	2.82	<0.01	4.16	0.69± 0.08	0.40	-
2	12.82	57.69	23.73±0.93[c]	20.51	3.65	61	<0.01	22.88	5.01±0.54[b]	3.29	<0.01	5.53	0.89± 0.10[c]	0.54	2
3	11.54	51.92	20.35±0.67	18.59	2.19	58	<0.01	14.95	1.95±0.28	0.91	<0.01	2.19	0.30±0.04	0.14	3
Bleomycin + Mitomycin C															
1	14.74	91.02	30.96±1.50[b]	26.28	7.30	71	<0.01	21.89	3.87±0.50[b]	2.17	<0.01	3.93	0.72±0.09	0.39	-
2	14.74	67.31	29.70±1.17[b]	26.28	4.58	82	0.01	27.10	5.28±0.56[b,d]	3.16	<0.01	4.17	0.86±0.09[c]	0.53	5
3	12.18	74.36	21.72±1.08	18.59	5.35	53	<0.01	9.99	1.90±0.23	0.91	<0.01	2.39	0.33±0.05	0.12	4
Control															
1	15.38	46.79	22.77±0.53[a]	17.63	0.46	5	<0.01	25.89	5.53±0.65[a]	1.81	<0.01	3.65	0.85±0.09[a]	0.28	-
2	12.82	30.77	18.10±0.29[b]	15.38	0.36	4	<0.01	9.86	2.53±0.24[b]	0.25	<0.01	1.39	0.38±0.04[b]	0.04	1
3	10.26	23.72	15.41±0.24	14.74	0.12	1	<0.01	5.83	0.83±0.13	0.11	<0.01	0.97	0.12±0.02	0.01	1

Data based on 100 comet measurements per sample. 1,2,3 - time of sampling; **1** – immediately after treatment; **2** – 24 hours after treatment; **3** – 48 hours after treatment. **H** – dispersion coefficient**; LTN** – Long-tailed nuclei; **A**-apoptosis.
Statistical significance of the results was evaluated on log-transformed data using ANOVA. Post-hoc comparisons were made using the Scheffé test. Significantly increased values (P<0.05) are marked with letters a-d. (**a**) Significantly higher than in samples 2 and 3; (**b**) significantly higher than in sample 3; (**c**) significantly higher than in samples 1 and 3; (**d**) significantly higher than in sample 1.

Table 2 *Results of the alkaline comet assay on control peripheral blood lymphocytes and lymphocytes treated with bleomycin (0.39 μg/mL), mitomycin C (0.31 μg/mL) and their combination in vitro for 24 hours. Antineoplastic drugs were added into the cell cultures after 24 hrs of lymphocyte growth* in vitro *(treatment B).*

	TAIL LENGTH / μm						TAIL DNA %				TAIL MOMENT				ΣA /100 cells
	Min.	Max.	Mean ±S.E.	Median	H	LTN	Min.	Max.	Mean ±S.E.	Median	Min.	Max.	Mean ±S.E.	Median	
Bleomycin															
1	15.38	85.90	29.93±1.49[a]	24.36	7.42	53	0.01	28.91	5.28±0.69[b]	2.02	<0.01	7.81	1.05±0.16[b]	0.36	8
2	12.18	35.26	16.60±0.38	16.03	0.85	8	<0.01	14.98	1.68±0.31	0.44	<0.01	3.46	0.26±0.05	0.07	-
3	12.82	142.30	28.03±2.76[c]	17.95	27.25	49	<0.01	29.19	4.43±0.61[b]	1.98	<0.01	16.28	1.02±0.24[b]	0.29	5
Mitomycin C															
1	20.51	78.20	34.11±1.08[c]	30.45	3.40	87	0.08	23.72	5.18±0.47[c]	4.13	0.02	5.63	1.02±0.10[c]	0.76	8
2	10.26	34.61	15.88±0.42	14.74	1.14	12	<0.01	18.58	1.98±0.35	0.47	<0.01	2.98	0.28±0.05	0.07	-
3	13.46	157.05	36.12±2.40[c]	29.17	15.99	85	<0.01	21.20	5.96±0.62[c]	2.90	<0.01	6.22	1.07±0.12[c]	0.56	5
Bleomycin + Mitomycin C															
1	21.79	98.72	40.11±1.59[c]	35.26	6.28	94	0.51	24.14	7.39±0.61[c]	5.46	0.11	6.50	1.43±0.13[c]	0.95	5
2	9.62	28.85	16.12±0.37	15.38	0.84	12	<0.01	9.81	1.51±0.23	0.38	<0.01	1.45	0.23±0.04	0.05	-
3	15.38	139.10	53.90±2.57[d]	50.96	12.25	97	<0.01	32.13	9.11±0.80[c]	6.69	<0.01	8.39	1.93±0.18[c]	1.39	-
Control															
1	11.54	28.85	17.37±0.28[a]	17.31	0.46	4	<0.01	4.94	1.20±0.13	0.75	<0.01	0.86	0.19±0.02	0.10	1
2	10.90	36.54	15.10±0.34	14.10	0.77	5	<0.01	7.94	0.87±0.16	0.20	<0.01	1.33	0.13±0.03	0.03	1
3	12.18	20.51	15.33±0.15	14.74	0.15	3	<0.01	8.00	0.85±0.14	0.17	<0.01	1.23	0.12±0.02	0.02	1

Data based on 100 comet measurements per sample. 1,2,3 - time of sampling; **1** – immediately after treatment; **2** – 24 hours after treatment; **3** – 48 hours after treatment. **H** – dispersion coefficient**; LTN** – Long-tailed nuclei; **A**-apoptosis.
Statistical significance of the results was evaluated on log-transformed data using ANOVA. Post-hoc comparisons were made using the Scheffé test. Significantly increased values (P<0.05) are marked with letters a-d. (**a**) significantly higher than in samples 2 and 3; (**b**) significantly higher than in sample 3; (**c**) significantly higher than in sample 2; (**d**) significantly higher than in samples 1and 2.

elsewhere using the alkaline comet assay (32, 33). However, the background levels of lymphocyte DNA damage were significantly modified after exposure to antineoplastic drugs.

Genotoxicity of bleomycin. Bleomycin induced high levels of primary DNA damage, both in resting and proliferative lymphocytes. The damage levels in treated lymphocytes were significantly higher than in control samples (Tables 1 and 2). The comparison showed significant differences between treatments A and B and no consistent pattern. However, the levels of DNA damage significantly decreased when treated lymphocytes were incubated for 24 hours in fresh growth media.

As shown in histograms (Figures 1 and 2) DNA damage substantially varied between treated cells. A right-hand shift in the distribution of comet parameters in bleomycin- treated cells is interpreted as an increase in DNA damage. The values of dispersion coefficient (H) for treated samples were also high. On the other hand, frequency distribution in control samples was more homogenous, and the H values were significantly lower (Tables 1 and 2).

Different studies confirmed that the onset of detectable damage in the alkaline comet assay depends on the mechanisms of action of DNA-damaging agents (34,35). In our investigation, the highest level of DNA damage was observed immediately after bleomycin treatment. Such a high positive response to the treatment with bleomycin was also observed in other studies (34-40). This may be due to a wide spectrum of primary DNA lesions, but also to indirectly induced intracellular oxidative stress leading to the formation of additional alkali-labile apurinic/apyrimidinic sites that are effectively detected by the comet assay.

A reduction in DNA damage observed in later measurements in our study points to DNA repair. Most

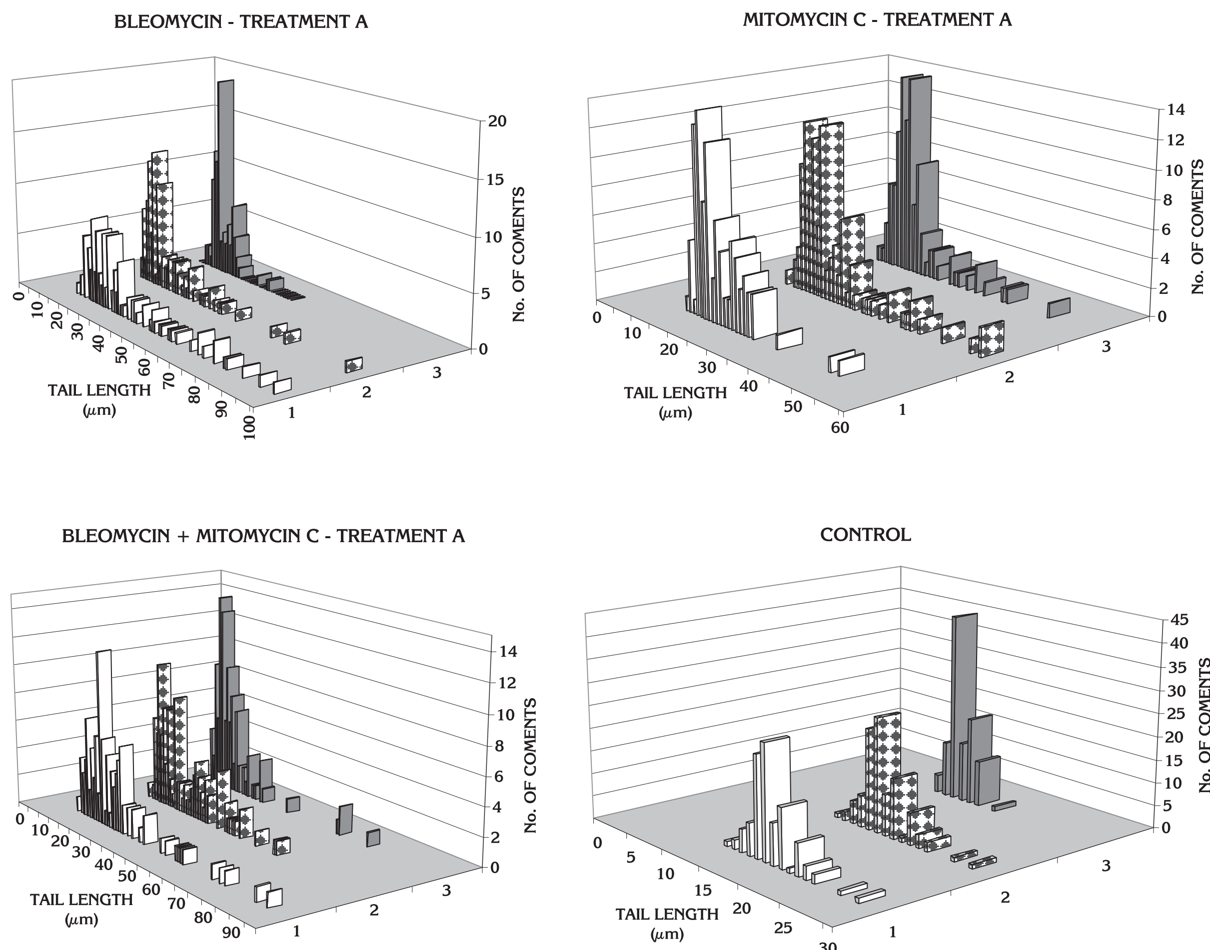

Figure 1 *Distribution of comet tail lengths in control lymphocytes and lymphocytes treated with bleomycin and/or mitomycin C for 24 hours. Antineoplastic drugs were added to cultures in the following concentrations: 0.39 μg/mL for bleomycin and 0.31 μg/mL for mitomycin C. Treatments lasted 24 hours. Antineoplastic drugs were added to cell cultures at the moment of culture initiation (treatment A). 1,2,3 – time of sampling; 1 – immediately after treatment; 2 – 24 hours after treatment; 3 – 48 hours after treatment*

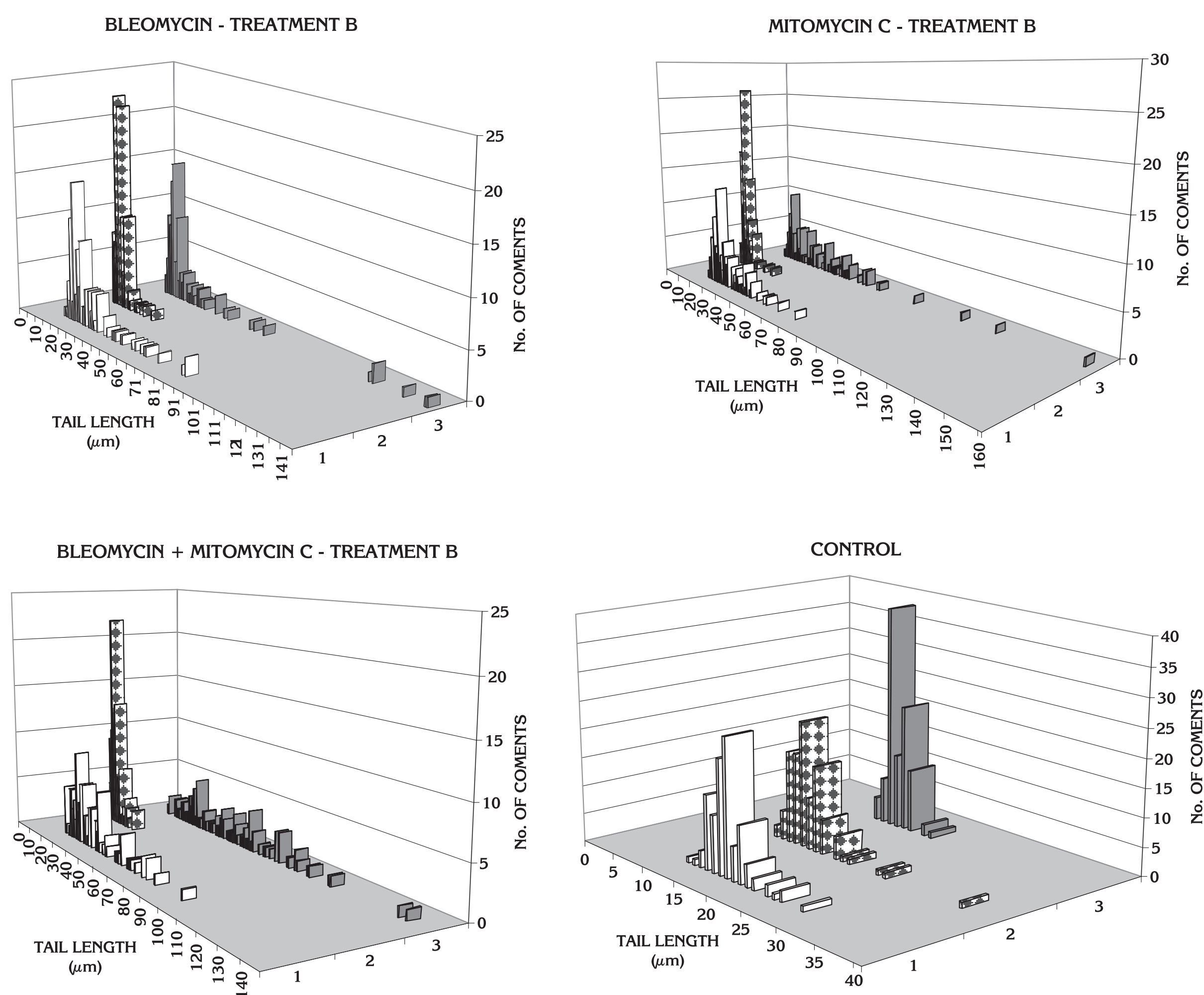

Figure 2 *Distribution of comet tail lengths in control lymphocytes and lymphocytes treated with bleomycin and/or mitomycin C for 24 hours. Antineoplastic drugs were added to cultures in the following concentrations: 0.39 μg/mL for bleomycin and 0.31 μg/mL for mitomycin C. Treatments lasted 24 hours. Antineoplastic drugs were added to cell cultures after 24 hrs of lymphocyte growth in vitro (treatment B). 1,2,3 – time of sampling; 1 – immediately after treatment; 2 – 24 hours after treatment; 3 – 48 hours after treatment.*

of the primary DNA lesions induced by bleomycin were effectively repaired 24 hours after the treatment (Tables 1 and 2). Similar observations have also been reported by other authors (38, 39, 41). The repair kinetics of treated lymphocytes in our study strongly depended on their proliferative status. The same was observed in other studies, indicating that mitogen stimulation of lymphocytes may result in an increased removal of specific types of DNA lesions. This observation might be explained by cell-cycle-dependent regulation of specific DNA repair enzymes, which are more active in proliferative than in resting cells (42, 43). During the post-treatment period, we also observed late effects of bleomycin exposure. They might be related to the depletion of the cell culture medium, production of clusters of lesions or multiply damaged sites (MDS) as well as to ineffective repair mechanisms. There is strong evidence that MDS have the potential for conversion to lethal lesions (44) while unrepaired DNA damage caused by bleomycin triggers apoptosis, that is, programmed cell death (9, 11). Similar has been observed in our study.

Genotoxicity of mitomycin C. In our study, mitomycin C induced different damage levels and different repair kinetics from bleomycin. The levels of primary DNA damage recorded in resting lymphocytes exposed to mitomycin C were significantly lower than in those treated with bleomycin and even lower than in corresponding control (Table 1). These observations are in accordance with earlier reports using standard alkaline comet assay which show that mitomycin C usually causes no increase or even decreased DNA migration (18, 23, 45).

In contrast, active cell proliferation significantly increases DNA migration and the levels of DNA damage. Although retarded DNA migration was

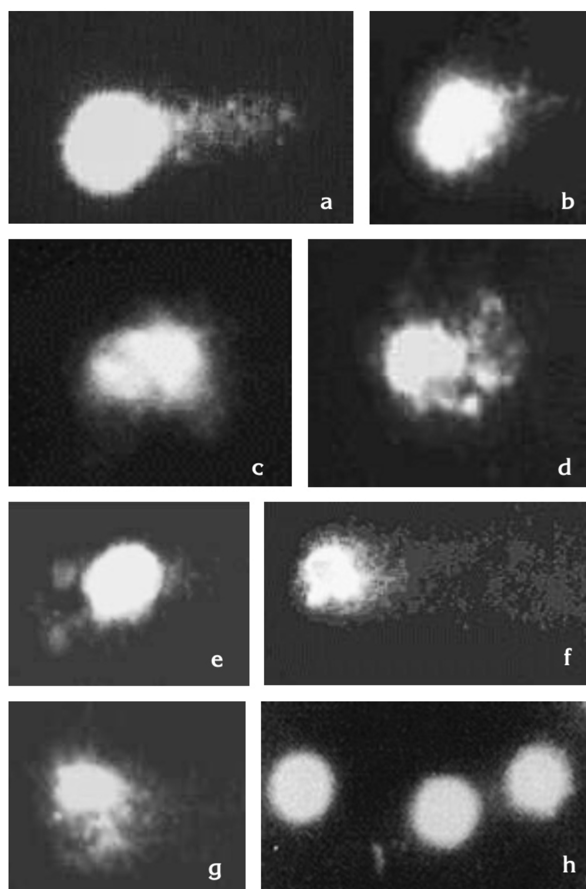

Figure 3 *Lymphocyte comets after in vitro exposure to bleomycin (BLM) or mitomycin C (MMC), or their combination. The concentrations were 0.39 μg/mL for BLM and 0.31 μg/mL for MMC. Treatments lasted 24 hours. (a) immediately after treatment A with BLM most comets had long tails containing a low fraction of highly fragmented DNA. (b) 48 hours after treatment B with BLM comet tails significantly decreased due to DNA repair. (c) immediately after treatment A with MMC most comets had short tails containing a high fraction of DNA. (d) 48 hours after treatment B with MMC various and untypical comet shapes were observed because DNA cross-linking prevented highly fragmented DNA from migrating during gel electrophoresis. (e) immediately after treatment A with BLM and MMC lymphocyte comets with untypical shapes were observed. (f, g) 48 hours after treatment B with BLM and MMC a number of comets with long tails containing a high fraction of DNA were observed. (h) Control lymphocytes had comets with very short tails or without any.*

recorded in resting lymphocytes, proliferative lymphocytes were more susceptible to mitomycin C. Immediately after treatment, their tail lengths, tail DNA %, tail moments, and the number of long tailed and apoptotic nuclei significantly increased (Table 2). Increased DNA migration in stimulated cells has also been reported by other investigators (42, 43).

Our results also confirmed earlier findings indicating that the repair of alkylated damage during the post-treatment period essentially contributed to DNA migration in mitomycin C-treated cells (Tables 1 and 2). When interstrand cross-links undergo nucleotide excision repair, additional breaks and alkali-labile sites are generated that are sensitively detected by the alkaline comet assay. Earlier reports show that mitomycin C-induced cross-links take at least 24 hours to repair completely and after their removal, cells displayed damage comparable to that shown by control cells (46). This study confirms these observations. Furthermore, our results are also consistent with reports (36) suggesting that cross-links induced by mitomycin C are not readily detected by the standard version of the alkaline comet assay because their migration-reducing effect is overwhelmed by the migration-inducing effect of other lesions. Therefore, the level of alkylation-induced damage as detected by the standard alkaline comet assay is a measure of DNA repair events, not of actual damage (36). Other authors reported that the repair of mitomycin C-induced lesions is often accompanied by a cell-cycle delay in S and/or G_2 phase (15,18). Interstrand cross-links caused by mitomycin C greatly distort the DNA structure and cause catastrophic consequences for a cell if unrepaired (47), and unrepaired DNA damage triggers apoptosis (15). In our study apoptotic nuclei were found after treatment with mitomycin C and their number was the highest in lymphocytes treated during active proliferation (Table 2).

Combined genotoxicity of bleomycin and mitomycin C. Combined treatment with both antineoplastic drugs showed a considerable variability in the pattern of lymphocyte DNA damage and repair. Our results suggest that bleomycin and mitomycin C synergetically induced DNA damage in peripheral blood lymphocytes (Tables 1 and 2). A comparison showed significant differences between treatments A and B. Depending on the position in the cell cycle, lymphocytes significantly differed in sensitivity to combined treatment. Moreover, the damage was significantly greater than in corresponding control cells (Tables 1 and 2). Adding both drugs to lymphocyte cultures produced different types of damage: single strand breaks, alkali labile sites and cross-links, but also a number of additional DNA alterations that might increase DNA migration. Resting lymphocytes simultaneously exposed to both drugs (treatment A) displayed significantly higher levels of primary DNA damage than those exposed to mitomycin C alone, but lower than those exposed to bleomycin alone (Table 1). This observation points to a possible retardation of DNA migration caused by the cross-linking activity

of mitomycin C in combined exposure. Proliferative lymphocytes (treatment B) were very susceptible to DNA damage caused by the combination of drugs (Tables 1 and 2). They displayed a significantly higher level of primary DNA damage than bleomycin and mitomycin C used alone (Table 2). Combined treatment also led to a high number of long tailed and apoptotic nuclei (Tables 1 and 2).

Our results point out that combined application of these drugs is highly effective in therapy, which is desirable in proliferative cancer cells, but not in non-cancer cells that unavoidably suffer severe genetic damage during and after chemotherapy. Our study also suggests that the kinetics of DNA repair strongly depends on lymphocyte proliferative status. The combined effects of bleomycin and mitomycin C are complex. It was reported earlier that the evaluation of the comet assay may be difficult where DNA lesions are accompanied by cross-links, since the test results will be a combination of inhibiting and inducing effects on DNA migration (48). The same was observed in our study in lymphocytes treated with both antineoplastic drugs.

The combined use of drugs, as opposed to monotherapy, has become a standard in modern chemotherapy of cancer. However, to achieve maximum therapeutic effect, these agents are used in high doses, and are often highly genotoxic to normal cells. Their ability to induce DNA damage in non-cancer cells may account for one of the most serious side effects produced by anticancer drugs – the induction of secondary malignancies (49). Although the primary DNA damage is subjected to efficient repair processes, some alterations remain and may contribute to the development of secondary malignancies unrelated to the original neoplasm (50). Because only some patients receiving a chemotherapy develop secondary cancer, it is important to determine whether they are hypersensitive to the treatment and whether this sensitivity may be indicative of the risk of the secondary cancer (3). For this reason, it is necessary to study certain biological markers that could be used as early predictors of disease progression and outcome. Since the effects of specific compounds are often tissue and cell-type related, it is important to develop, validate and apply techniques that can detect DNA damage at the single-cell level (30). The alkaline comet assay seems to fulfil this purpose.

CONCLUSION

This study has achieved its aim and we were able to demonstrate the correlation between the alkaline comet assay and the response to treatment. The results obtained suggest that therapeutic concentrations of bleomycin and mitomycin C applied *in vitro* alone and in combination can significantly increase primary DNA damage in human lymphocytes. The analysis of DNA repair kinetics confirmed different patterns of DNA damage when the drugs were used alone and in combination. We also observed significant differences in DNA damage induction and repair with respect to the lymphocyte proliferative status and their position in the cell cycle. It is quite possible that similar levels of DNA damage are also induced in non-cancer cells of patients who received bleomycin and mitomycin C during chemotherapy. However, the pharmacokinetics of antineoplastic drugs is different *in vivo*; they do not fully bind or interact with DNA and the drug metabolism in the liver also affects its action.

Despite limitations, our results show that the alkaline comet assay is a useful screening method for *in vitro* studies of drug-DNA interactions, especially for studying mechanisms of action of new drugs.

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Sažetak

PROCJENA GENOTOKSIČNOSTI BLEOMICINA I MITOMICINA C METODOM KOMETNOG TESTA

Većina antineoplastičnih lijekova ima nespecifično djelovanje pa su na njihovu primjenu osjetljive i stanice zdravog tkiva. U pretkliničkim istraživanjima lijekova važnu ulogu imaju brzi i osjetljivi testovi za procjenu razina oštećenja i popravka stanične DNA. Cilj istraživanja bio je procijeniti toksičke učinke bleomicina i mitomicina C na genom ljudskih limfocita u uvjetima *in vitro* primjenom kometnog testa u alkalnoj izvedbi. Limfociti su 24 h izlagani pojedinačnim lijekovima te njihovoj kombinaciji u terapijskim koncentracijama, a potom je praćena dinamika oštećenja i popravka DNA. Postavljena su dva pokusa: (A) u kojem su lijekovi dodavani u trenutku uspostavljanja staničnih kultura kako bi se procijenili toksički učinci na limfocite u fazi mirovanja i početnim fazama rasta, i (B) u kojem su lijekovi dodavani nakon 24 h rasta stanica u kulturi, kako bi se istražili učinci na stanice u diobi. Rezultati istraživanja pokazuju da oba lijeka izazivaju značajna primarna oštećenja limfocitne DNA. Razine oštećenja i dinamika njihova popravka ovisne su o diobenoj aktivnosti limfocita i njihovu položaju unutar staničnog ciklusa. Oštećenja izazvana bleomicinom vrlo su jaka neposredno nakon tretmana, a u kasnijim fazama rasta limfocita većinom se popravljaju. Mitomicin C izaziva drukčiji obrazac oštećenja i popravka DNA. Zbog ukriženog povezivanja između lanaca DNA, on značajno usporava migraciju DNA tijekom elektroforeze u alkalnim uvjetima, a visoke razine oštećenja koje se detektiraju u kasnijim fazama rasta uzrokovane su procesima popravka DNA. Bleomicin i mitomicin C sinergistički izazivaju opsežna oštećenja limfocitne DNA, osobito u diobeno aktivnim stanicama. Dobiveni rezultati govore u prilog primjene kometnog testa u pretkliničkim istraživanjima antineoplastičnih lijekova i upućuju na moguću primjenu ove metode u procjeni oštećenja genoma proizašlih iz izloženosti ovim agensima.

KLJUČNE RIJEČI: *antineoplastični lijekovi, kemoterapija, oštećenja i popravak DNA*

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