Cytogenetic Damage in Circulating Lymphocytes and Buccal Mucosa Cells of Head-and-neck Cancer Patients Undergoing Radiotherapy

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This study evaluated cytogenetic damage by measuring the frequency of micronucleated cells (MNC) in peripheral blood and buccal mucosa of head-and-neck cancer patients undergoing radiotherapy.

MNC frequencies were assessed in 31 patients before, during, and after radiotherapy, and in 17 healthy controls matched for gender, age, and smoking habits. Results showed no statistically significant difference between patients and controls prior to radiotherapy in cytokinesis-blocked lymphocytes or buccal mucosa cells. During treatment, increased MNC frequencies were observed in both cell types. Micronucleated lymphocyte levels remained high in samples collected 30 to 140 days after the end of treatment, while MNC frequency in buccal mucosa decreased to values statistically similar to baseline values. There is controversy over the effects of age, smoking habit, tumor stage, and/or metastasis on MNC frequency. However, increased frequency of micronucleated buccal mucosa cells was seen in patients under 60 years old and in those with tumors >4cm.

In conclusion, the data show that radiotherapy has a potent clastogenic effect in circulating lymphocytes and buccal mucosa cells of head-and-neck cancer patients, and that the baseline MNC frequency in these two tissues is not a sensitive marker for head-and neck neoplasm.

INTRODUCTION

Radiation plays a key role in the treatment of many neoplasias. However, it is well known that ionizing radiation damages DNA, including single- and double-strand breaks, base damage, and DNA-protein cross links. As a consequence, a second tumor may develop immediately or years after the primary tumor treatment.[1,2,3] Attempts have been made to evaluate the genotoxicity of ionizing radiation in patients undergoing radiotherapy. Gamma rays have been reported as inducing linear increase of micronucleated buccal mucosa cells in oral cancer patients undergoing radiotherapy,[4] and increasing the frequency of micronuclei in peripheral blood lymphocytes of patients with malignancies in different sites.[5,6,7,8] The effects of radiation on the DNA of a living organism can be studied in individual cells (e.g.,

chromosome aberration, micronucleus, cell killing and transformation), and DNA molecules (e.g., primary lesions, DNA repair mechanisms). Simultaneous analysis of micronuclei in cytokinesis-blocked peripheral blood lymphocytes, as developed by Fenech and Morley,[9] and exfoliated cells are increasingly being applied to monitoring human exposure to mutagens.[10,11] However, validating micronuclei as a biomarker linking environmental exposure to human disease is still limited because of the wide variation in frequency estimation among laboratories.[12]

Chromosome analysis has shown a high incidence of numerical and structural abnormalities in cancer patient tumor tissue samples and peripheral blood lymphocytes.[13,14] In this study we investigated whether micronucleated lymphocytes or micronucleated buccal mucosa cells can be sensitive markers for the presence of head-and-neck cancer, and for the mutagenic effect of radiation in head-and-neck cancer patients during and after treatment.

MATERIALS AND METHODS

Subjects

The study populations included a group of 31 head-and-neck cancer patients (25 male, 6 female) without any prior

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Table 1. General characteristics of the study populations.

Gender	Age (years)	No. cigarettes/ day	Tumor characteristics			EQ dose (Gy)	
			Type	Site	Stage	Mid-treatment ($3^{rd} - 4^{th}$ week)	Cumulative (post-treatment)
Patients							
M	76	20	SCC	Oropharynx	III ($T_3N_0M_0$)	5.00	5.50
M	50	40	SCC	Tongue	III ($T_2N_1M_0$)	4.86	6.04
M	51	20	SCC	Buccal mucosa	III ($T_3N_1M_0$)	2.16	6.84
F	37	3	SCC	Rhino pharynx	III ($T_3N_1M_0$)	5.24	7.04
M	67	2	SCC	Larynx	III ($T_3N_0M_0$)	3.42	NR
M	72	20	SCC	Retromolar	I ($T_1N_0M_0$)	3.24	NR
F	43	6	CAC	Hard palate	IV ($T_4N_0M_0$)	2.34	4.50
M	69	20	SCC	Pyriform sinus	III ($T_2N_1M_0$)	2.70	5.94
M	63	40	SCC	Larynx	II ($T_2N_0M_0$)	3.60	6.04
F	51	10	SCC	Tonsil	IV ($T_4N_3M_0$)	4.00	7.80
M	57	0	SCC	Larynx	IV ($T_4N_0M_0$)	2.52	6.04
M	69	30	SCC	Floor of mouth	IV ($T_xN_2M_0$)	2.70	6.48
M	66	20	CAC	Nasal cavity	IV ($T_4N_0M_0$)	3.40	NR
M	59	20	SCC	Tongue	I ($T_1N_0M_0$)	4.14	6.00
M	44	30	SCC	Floor of mouth	IV ($T_4N_0M_0$)	1.14	6.04
M	76	0	SCC	Gingiva	III ($T_2N_1M_0$)	1.62	NR
F	65	0	MLN	Cervical metastasis	–	2.80	5.60
M	61	60	SCC	Larynx	I ($T_1N_0M_0$)	5.20	6.60
M	58	80	SCC	Larynx	IV ($T_4N_0M_x$)	3.60	6.70
F	65	20	CAC	Hard palate	IV ($T_4N_0M_0$)	2.34	6.64
M	54	20	SCC	Hypopharynx	IV ($T_4N_0M_0$)	1.98	5.40
M	78	40	SCC	Supra glottic	II ($T_2N_0M_0$)	2.70	7.04
M	55	40	SCC	Floor of mouth	IV ($T_2N_{2b}M_0$)	4.50	6.44
M	52	30	SCC	Supra glottic	III ($T_3N_0M_0$)	3.24	6.12
M	47	40	SCC	Tonsil	III ($T_3N_1M_0$)	4.86	6.04
M	56	80	SCC	Retromolar	II ($T_2N_0M_0$)	4.68	6.04
M	57	20	SCC	Soft Palate	IV ($T_4N_0M_0$)	3.06	5.98
M	68	20	SCC	Floor of mouth	IV ($T_4N_{2b}M_0$)	2.70	6.04
F	44	40	SCC	Orofarynx	III ($T_3N_1M_0$)	1.98	6.04
M	54	30	SCC	Larynx	IV ($T_4N_{2a}M_x$)	3.96	6.48
M	73	40	SCC	Tongue	IV ($T_4N_1M_0$)	1.98	6.04
25M; 6F	59.3 ± 10.8^1	27.1 ± 20.4				3.28 ± 1.13	6.18 ± 0.65
Controls							
M	46	10					
M	75	10					
F	34	20					
F	46	20					
M	76	10					
M	55	30					
M	42	30					
M	46	20					
M	55	20					
M	58	40					
M	48	20					
M	40	20					
M	49	10					
F	45	10					
M	55	20					
M	73	40					
M	53	20					
14M; 3F	52.7 ± 12.1	20.6 ± 9.7					

[1] Mean ± SD; EQ dose, equivalent body dose; SCC, squamous cell carcinoma; CAC, carcinoma adenoid cystic; MLN, melanoma; NR, the patient did not return for post-treatment analysis.
TMN (Tumor-Node-Metastases) System, adopted by the American Joint Committee on Cancer and the International Union Against Cancer.

treatment (radiotherapy and/or chemotherapy), and a group of 17 healthy volunteers (14 male, 3 female) matched for age without occupational exposure to environmental and/or medical genotoxic agents. All control individuals were smokers since most patients were also smokers (28/31). Table 1 shows population general characteristics, such as gender, age, number of cigarettes/day, and the type, site, and stage of the tumor according to TMN (Tumor-Node-Metastases) System. A questionnaire on individual lifestyle (e.g. occupation, tobacco, and alcohol) and pathological status (type, location, and stage of tumor), and signed informed consent were obtained from each subject. The Botucatu School of Medicine Ethics Committee, UNESP, SP, Brazil approved the protocol.

Radiation treatment of cancer patients was performed using a 4 or 6MeV linear accelerator (X-ray). Doses ranged from 45 to 70.4Gy, delivered in daily fractions of 1.8–2.0Gy, 5 days per week for 5–7 weeks. Doses are expressed as equivalent body dose, estimated by dividing the integrated dose by body weight.

Micronucleus assay for lymphocytes

Peripheral blood (5ml) was collected from each cancer patient at three different times: 1) just before start of radiation treatment, 2) during the radiotherapy (between the 3rd and 4th weeks), and 3) 30–140 days after the completion of treatment. A single sample was collected from controls by venipuncture using a heparinized syringe (Liquemine, 5,000UI/ml; Roche). Two independent lymphocyte cultures were set up for each blood sample[15] with 0.5ml whole blood added to 6ml RPMI-1640 medium (Cultilab, Brazil) containing 2ml fetal calf serum (Cultilab) and 2% phytohaemagglutinin A (Cultilab). Cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 44h, cytocalasin B

(Sigma, St. Louis, MO; 4.5 µg/ml final concentration) was added, and the cells were allowed to grow for another 28h. Cells were then harvested by centrifugation, treated with 0.075M KCl cold hypotonic solution, and fixed in 5:1 methanol/acetic acid. The cells were dropped onto pre-cleaned coded slides, air-dried, and stained with fresh 5% Giemsa solution for 7 min. From each patient, 1,000 binucleated lymphocytes (500 cells from each culture) with well-preserved cytoplasm were blindly scored by light microscope (400X). The results were expressed as frequency of micronucleated binucleated lymphocytes. Nuclear division index (NDI) was also determined by scoring the number of nuclei in 400 cells: NDI = $[M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]$ / N, where M_1 to M_4 represent the number of cells with one to four nuclei, respectively, and N is the total number of cells scored.[16]

Micronucleus assay for exfoliated buccal mucosa cells

Exfoliated buccal mucosa cells were collected concomitantly with blood samples (before, during, and after radiation). After rinsing the mouth with tap water, cells were obtained by scraping the cheek mucosa with a moist wooden spatula. For head-and-neck cancer patients, separate samples were collected from each side of the mouth. The cells were transferred to a tube containing saline solution, centrifuged (800rpm), fixed in 3:1 methanol/acetic acid, and dropped onto pre-cleaned slides, air-dried, and stained with the Feulgen/Fast-Green method; they were examined under light microscope (400X) to determine MNC frequency. Two thousand cells (1,000 cells from each side of the cheek) were scored from each patient, for the first and third cell sampling times (before and after radiotherapy, respectively), and 4,000 cells were scored (2,000 cells from each side of the cheek) at the second sampling time (during radiotherapy). For con-

Table 2. Frequency of micronucleated cells (MNC; lymphocytes and buccal mucosa cells) in healthy subjects (control) and cancer patients distributed according to the tumor stage (1,000 cells/individuals).

Groups	Lymphocytes		Buccal mucosa	
	Individuals	MNC (‰) Mean ± SD	Individuals	MNC (‰) Mean ± SD
Control	15	3.14 ± 2.74	17	0.50 ± 0.61
Patients prior to treatment[a]	31	2.16 ± 1.59	31	0.79 ± 1.05
Tumor stage[b]				
I and II[c]	6	1.71 ± 2.06	6	0.42 ± 0.80
III	10	2.40 ± 1.71	10	0.77 ± 0.75
IV	14	2.21 ± 1.31	14	0.96 ± 1.34

[a] All patients together; [b] one patient was excluded because the tumor stage was not classified (melanoma and cervical metastasis); [c] Tumor stages I and II were grouped because there were few individuals in each group.

Table 3. Frequency of micronucleated cells (MNC; binucleated lymphocytes and buccal mucosa cells) in cancer patients undergoing radiotherapy.

Groups	Lymphocytes		Buccal mucosa	
	Individuals	MNC (‰) Mean ± SD	Individuals	MNC (‰) Mean ± SD
Prior to treatment	25	1.88 ± 1.62[a]	26	0.79 ± 1.03[a]
Mid-treatment	25	28.88 ± 16.02[b]*	26	2.29 ± 1.38[b]*
Post-treatment	25	32.68 ± 24.73[b]*	26	1.67 ± 2.15[a]

[a,b]Different letters indicate statistically significant differences between groups; * $P < 0.01$.

trols, cells were sampled only once, and 2,000 cells were scored from each individual. Micronuclei were scored according to the criteria described by Sarto *et al.*.[4]

Statistical analysis

The Friedman or Wilcoxon test for dependent samples were used to compare MNC frequency between samples (before, during, and after radiation). For differences between groups, pair-wise multiple comparisons were made using the Student-Newman-Keuls method. The Kruskal-Wallis or Mann-Whitney test was used to compare differences between cancer patients and healthy subjects, or other independent parameters (tobacco habits, age, tumor characteristics, radiation-dose rank). The Spearman test was used for correlation between MNC frequency in peripheral blood and buccal mucosa. $P < 0.05$ was considered significant. Patients who died during the treatment period or moved to another Radiotherapy service were excluded.

RESULTS

MNC in healthy controls and cancer patients before radiotherapy (Table 2)

As there was no detectable difference in buccal mucosa MNC frequency between both cheeks of each cancer patient (data not shown), data were pooled. The MNC basal frequency (‰) in patients before therapy, independent of tumor site or stage, was 2.16 ± 1.59 in blood and 0.79 ± 1.05 in buccal mucosa. There was no significant difference between blood and buccal mucosa in the controls (3.14 ± 2.74 and 0.50 ± 0.61, respectively). There was also no difference between MNC frequencies in patients with different tumor stages.

MNC frequency in cancer patients treated with fractionated partial-body radiotherapy

A statistically significant increase in micronucleated lymphocytes was seen during (28.88 ± 16.02) and after (32.68 ± 24.73) radiation exposure compared to baseline pre-therapy samples (1.88 ± 1.62). In buccal mucosa cells a significant difference was only seen mid-treatment (2.29 ± 1.38 vs. 0.79 ± 1.03 prior to therapy; Table 3). For the Friedman test,

paired data were obtained from 25/31 (lymphocyte) and 26/31 (buccal mucosa) patients once cells had been collected from all 3 sampling times (pre-, mid-, and post-treatment).

To evaluate radiation dose-effect during and after (cumulative dose) treatment, patients were distributed into 3-dose and 2-dose groups, respectively Fig. 1 shows mid-treatment results for the three dose groups: <2.6Gy (1.14–2.34Gy), 2.6G ≤ 3.5Gy (2.70–3.42Gy), and >3.5Gy (3.60–5.20Gy).
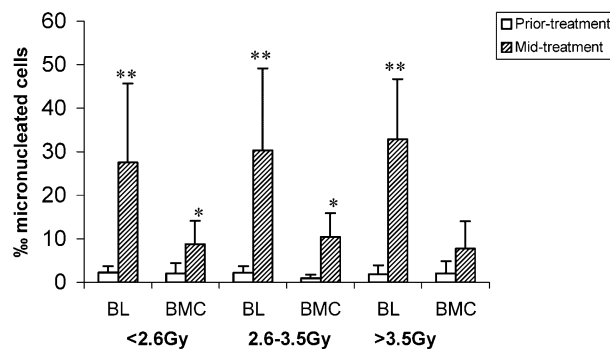

Fig. 1. Frequency of micronucleated cells in head-and-neck cancer patients undergoing radiotherapy (mid-treatment). BL, binucleated lymphocytes; BMC, buccal mucosa cells; * $P < 0.05$; ** $P < 0.01$.
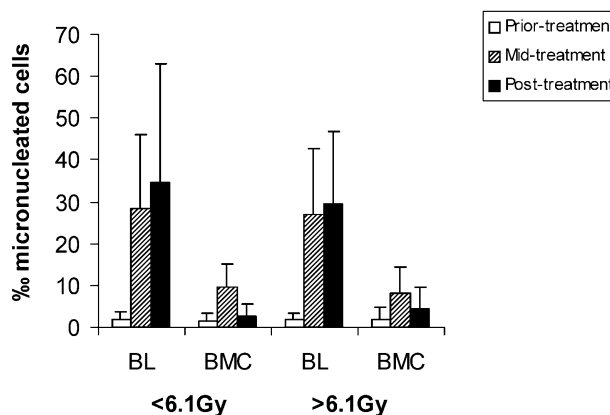

Fig. 2. Frequency of micronucleated cells in head-and-neck cancer patients following radiotherapy grouped according to the cumulative dose of radiation. BL, binucleated lymphocytes; BMC, buccal mucosa cells

Although micronucleated lymphocyte frequency was higher in the three groups, there was no significant difference among them. In buccal mucosa, increased MNC frequencies were seen in the lowest and intermediate groups compared to before treatment (baseline value). Fig. 2 shows MNC frequencies in patients distributed into two cumulative dose ranges: <6.1Gy (4.50–6.04Gy), and >6.1Gy (6.12–7.8Gy). There were significant increases in micronucleated cells for peripheral blood, but not for buccal mucosa, in both groups compared to the baseline pre-therapy samples. However, no

Table 4. Distribution of micronuclei in binucleated lymphocytes of head-and-neck cancer patients undergoing different doses of radiotherapy.

Radiotherapy dose (Gy)	MNC[a] (‰)	No. of micronuclei per binucleated cell					Total no. of micronuclei	Micronuclei/ 1000 cells	NDI[b]
	Mean ± SD	0	1	2	3	4			
Prior–treatment (n=31)	2.16 ± 1.59	30933	67	0	0	0	67	2.16 ± 1.59	1.54
Mid-treatment									
< 2.6 (2.0 ± 0.4) (n=9)	27.56 ± 18.06	8713	258	23	5	1	323	35.89 ± 20.74*	1.53
2.6–3.5 (3.0 ± 0.3) (n=10)	30.20 ± 18.92	9628	323	43	2	4	431	43.10 ± 30.37*	1.35
> 3.5 (4.5 ± 0.6) (n=10)	32.90 ± 13.83	9581	366	51	2	0	474	47.40 ± 22.03*	1.44
Post-treatment									
<6.1 (5.8 ± 0.4) (n=16)	34.56 ± 28.38	15294	558	124	14	10	888	55.00 ± 50.76*	1.43
>6.1 (6.7 ± 0.4) (n=11)	29.73 ± 15.77	10502	338	48	16	4	498	45.30 ± 31.65*	1.19*

[a]Micronucleated cells; [b]Nuclear division index = [M1 + (2 X M2) + (3 X M3) + (4 X M4)] / N, where M1- M4 are the number of cells with 1 - 4 nuclei, and N is the total number of cells scored. * $P < 0.01$.

Table 5. Analysis of factors potentially affecting the frequency of micronucleated cells (MNC) in peripheral blood and buccal mucosa from cancer patients prior to radiotherapy.

Factor	Groups	Number of patients		MNC (‰)		P value
		BL	BMC	BL	BMC	
Age	< 60	17	17	2.29	1.45*	< 0.05
(years old)	> 60	14	14	2.00	0.36	
Tobacco habits	≤ 20	17	17	2.35	0.76	> 0.10
(cigarettes/day)	≥ 30	14	14	1.93	0.82	
Tumor stage	I + II[a]	6	6	2.00	0.42	
	III	10	10	2.40	0.77	> 0.10
	IV	14	14	2.20	0.96	
Tumor size	$T_1 + T_2$[a]	10	10	2.10	0.35	
	T_3	7	7	2.57	1.06**	< 0.05
	T_4	12	12	2.00	1.04	
Presence of regional metastasis	No	17	17	2.18	0.70	> 0.10
	Yes	13	13	2.31	0.89	

[a]Tumor stages I and II, and tumor sizes T_1 and T_2 were grouped because there were few individuals in each group; BL, binucleated lymphocytes; BMC, buccal mucosa cells; * (<60) > (<60); **$T_3 > T_1 + T_2$; $T_3 = T_4$.

difference was detected between the two dose levels ($P <$ 0.01).

Table 4 shows the distribution of micronuclei per binucleated lymphocyte and NDI. Before treatment all MNC had only one micronucleus (MN); during and after radiotherapy, MNC presented up to four micronuclei. There were significant differences in MN frequency between before and after radiotherapy, but no difference between doses. NDI significantly decreased in >6.1Gy doses.

Effect of age, tobacco status, and tumor stage on baseline MNC frequency

Prior to radiotherapy, there was significant difference in MNC frequency in both tissues between patients smoking <20 or >30 cigarettes per day. However, MNC frequency in buccal mucosa was higher in the under-60 group, and in those with T_3 (tumor size >4cm) tumors (Table 5). No correlation was found between tumor size and tumor stage classified by the TMN (Tumor-Node-Metastases) System.

DISCUSSION

Micronuclei represent chromosome fragments or whole chromosomes which are not incorporated into the main nuclei at mitosis and consequently they appear only in cells that have undergone nuclear division.[9] Based on these concepts and that chromosome alterations are important events in cancer development, we used the lymphocyte cytokinesis-block MN assay and MN test in exfoliated buccal mucosa cells to assess chromosome damage in head-and-neck cancer patients prior to any treatment, and during and after radiotherapy.

Increased MN and chromosome damage frequency have been seen in patients with different cancers,[6,14,17] in families with high incidences of cancer,[8] and in proliferating basal cells from normal tissues surrounding head-and-neck pre-neoplastic and malignant lesions.[18] However, our data did not show significant difference in MN frequency between cancer patients and healthy individuals for both lymphocytes and buccal mucosa cells.

Different confounding factors, such as gender, age, and personal habits must be considered in human cytogenetic studies. Alterations in the immune system, and defects in DNA repair system[6,19] have been associated with increased frequencies of chromosome aberrations in cancer patients prior to therapy. Tobacco status is also usually considered in analyses. A recent study has shown significant difference in MNC frequency between non-smoker cancer patients and non-smoker healthy subjects. However, no significant difference was detected when both groups were smokers.[20] Our results agree with these findings, as there was no significant difference between head-and-neck cancer patients (91% smokers) and healthy controls (100% smokers). Also, there was no significant difference in the MNC frequency between

patients who smoked less than 20 cigarettes per day and those smoking 30–80 cigarettes per day. Similar data have been reported in other studies.[8,21] According to Duffaud *et al.*,[20] pathological status could mask the smoking effect on peripheral blood lymphocytes in cancer patients, and conversely, the effects of smoking may mask the significance of MNC frequencies on cancer risk. Moreover, these authors suggest that the cytokinesis-block lymphocyte MN assay is a sensitive method for detecting chromosome alterations in non-smokers, but is not recommended for studying DNA damage in smoking patients, especially for those with upper aero-digestive tract cancers.[20]

In our study variables such as tumor stage, and presence of metastases had no effect on MNC frequency prior to radiotherapy. Although some authors have reported no effect from gender on MNC frequency,[5,6,20] samples in this study were not suitable for drawing such a conclusion, since there were only 6 females, and tobacco consumption by the 25 male (mean = 30.5 ± 20.4 cigarettes/day), and 6 female (mean = 13.2 ± 14.9 cigarettes/day) patients was different. In exfoliated buccal mucosa cells, we observed that the tumor size (T) interfered in MNC frequency, although in some cases the cells sampled were not proximal to the cancerous lesion. Patients with T_3 tumors (tumor size >4cm) showed slightly increased MNC in buccal mucosa compared to those with T_1 or T_2 (but not T_4) tumors, although there was no correlation with the tumor stage. The published data regarding the age influence on the frequency of micronucleated cytokinesis-blocked lymphocytes are conflicting.[6,9,20,22] Although a statistically significant effect of age on buccal mucosa cells (but not on lymphocytes) was observed in the present study, this result could be due to the difference in the proportion of patients T_3 and T_4 between the ones under 60 years old and those over 60.

The mid-treatment samples from cancer patients undergoing radiotherapy showed no dose-dependent increase in MNC frequencies (either in blood or buccal mucosa) compared with their own concurrent pre-treatment samples. Increased frequencies of micronucleated lymphocytes were also detected after the completion of radiotherapy; this was seen only in relation to baseline data (prior to treatment), and not to frequencies during treatment. Similarly to mid-treatment, no dose effect was seen when patients were grouped into two cumulative dose levels (<6.1Gy, and >6.1Gy). An increase and subsequent reduction in micronucleated lymphocyte frequency has been described in patients undergoing fractionated radiotherapy and in *in vitro* studies with irradiated blood samples.[23,24,25] Ramalho *et al.*[25] have observed that at high doses (like 400rads) the capacity to detect fragments by MN technique decreases to 38–45%. This low efficiency has also been seen even at higher doses, i.e. 6 rads and 800rads (6cGy and 8Gy). These authors have also observed that the yields of dicentric chromosomes and micronuclei do not increase in a linear or quadratic manner

after 600rads, but reach a near saturation effect. Based on this, the authors have suggested that the effect on micronuclear yield may be due to the highly affected cells not entering mitosis and more than one fragment fusing to form a single MN.[25] Similar data have been observed after *in vitro* X-ray exposure.[24] Reduced numbers of MN per unit dose compared to doses below 5Gy, and a decline in MN frequency after doses exceeding about 10Gy have been described.[24] When the NDI and distribution of micronuclei were evaluated, we did not observe any difference among radiation doses in mid-treatment. A significant decrease in NDI was seen for cumulative doses higher than 6.1Gy. These findings suggested that cytotoxicity (cell death or cell cycle delay) could have interfered in the frequency of radiation-induced MNC weeks after the end of the irradiation, and could have masked a possible dose-effect relationship.

Regarding MNC frequency in buccal mucosa, differences were found between pre- and mid-treatment, and between mid- and post-treatment. The post-treatment result (30 to 140 days after; mean 64.9 ± 28.0 days) showed that MNC frequency decreases to values that are not statistically different from the baseline levels. Similar results have been described in patients suffering from cancer of the oral cavity and undergoing mouth radiotherapy. Within 7 to 12 days after the end of radiotherapy (2000rads), MNC frequency in buccal mucosa was reduced to the initial background values.[4] This reduction was attributed to the cytotoxic effect of radiation leading to a loss of heavily damaged cells in the basal dividing cell layer, and increased cell death in the surface layer.[4] It is usually assumed that MN frequencies are higher in the basal layer, where they are generated, than in more superficial and exfoliated cells.[26] Moore *et al.*[27] have observed an increased MN rate after 3 and 6 weeks of photon radiation therapy, but three weeks after therapy cessation, the rate returned close to baseline levels.

In conclusion, our data confirm that radiotherapy has a potent clastogenic effect on circulating lymphocytes and buccal mucosa cells of head-and-neck cancer patients. We believe that these results can be used in pooled analyses for evaluating the side effects of radiotherapy, and to contribute to the MN database for understanding and improving assays.

ACKNOWLEDGMENTS

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