Relationship between Genotoxic Effects of Breast Cancer Treatments and Patient Basal DNA Integrity

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ABSTRACT: Radiotherapy and chemotherapy cause genotoxic side effects that are highly variable among patients. In this study, we evaluated DNA integrity using the comet assay in peripheral blood lymphocytes from breast cancer patients before ("pre-treatment patients"; n = 47) and after ("post-treatment patients"; n = 24) radiotherapy and/or chemotherapy treatment and from healthy donors (n = 15). Comet evaluation was made by visual (types 0–4) and digital (percentage of DNA remaining in the comet head = % head DNA) analysis. The association between the level of DNA damage and cancer prognostic factors was assessed. The treatments caused a significant increase in DNA damage registered by both visual ($p < 0.001$) and digital ($p < 0.001$) analyses. No significant associations between the level of DNA damage in pre-treatment patients and cancer prognostic factors were found. A significant correlation between the comet results from each patient before and after treatment ($r = 0.64$, $p = 0.001$) was observed. The % head DNA in post-treatment samples from patients with a high level of DNA damage before treatment ($30.3 \pm 3.1\%$, $p < 0.01$) was lower than in post-treatment samples from patients with a low-to-medium level of DNA damage before therapy ($49.2 \pm 4.4\%$). These results support the usefulness of the comet assay as a sensitive technique to evaluate basal DNA status and DNA damage caused by cancer treatments. The comet assay could contribute to treatment decisions, especially by taking into account the patient's basal DNA damage before therapy.

KEY WORDS: comet assay, genotoxic damage, peripheral blood lymphocytes, prognostic factors

I. INTRODUCTION

Exposure to genotoxic agents during chemotherapy or radiotherapy can produce a wide variety of side effects in human health.[1] The efficiency of radiotherapy is limited by the adverse side effects in normal tissues when exposed to radiation. Generally, more than 5% of breast cancer patients develop acute or late symptoms of enhanced radiosensitivity.[2] Acute side effects, such as erythema and desquamation of the exposed skin and mucosa, occur during or shortly after therapy, whereas depending on elapsed time, late effects can vary from severe tissue alterations (e.g., fibrosis and telangiectasia) to secondary malignancies.[3] Furthermore, after therapeutic exposure to ionizing radiation, different levels of damages can be identified at the nuclear DNA level.[2]

In addition to the beneficial actions of chemotherapy, the adverse consequences of its action on normal tissues are observed very frequently. Anti-tumor drugs generally do not selectively affect tumor-cell DNA.[4] Indeed, following *in vivo* exposure to antineoplastic drugs, diverse lesions in DNA are induced.[5] In fact, several studies have shown that

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patients with a successful response to chemotherapy have a higher risk of developing secondary cancers.[6]

Decisions regarding the use of adjuvant therapy are strongly influenced by the risk of disease recurrence and death. These risks are assessed by examining prognostic factors of breast cancer, such as axillary lymph-node metastasis, age, and some tumor characteristics (e.g., size, histologic and nuclear grades, and estrogen and progesterone receptor status). Regarding these factors, the presence of node metastasis, lower age at clinical detection, larger tumor size, higher histologic and nuclear grades, and absence of hormone receptors are markers of poor prognosis.[7]

DNA damage caused by anti-neoplastic drugs and ionizing radiation includes nucleotide damage, breaking of hydrogen bonds between the two helices, single-strand breaks, double-strand breaks, appearance of apurinic or apyrimidinic sites, DNA protein cross-links, DNA–DNA interstrand cross-links, DNA intrastrand cross-links, and generation of reactive oxygen species, which, in turn, cause more DNA damage.[2,8,9]

DNA damage related to the exposure of cancer patients to therapy is frequently determined using peripheral blood lymphocytes as target cells. Lymphocytes have been proven to be good surrogate cells with which to investigate DNA damage because they are affected by agents used in cancer treatment and also are easy to obtain.[8,10]

Diverse methodological approaches have been developed to assess the genotoxic damage. Among them, the comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA integrity in individual cells. The alkaline version of the assay allows to detect DNA single- and double-strand breaks, alkali-labile sites (e.g., apurinic and apyrimidinic sites), DNA–DNA and DNA–protein cross-linking, and single-strand breaks associated with incomplete excision repair sites.[11,12] In recent years, the comet assay has become an important tool in genetic toxicology.[10,13]

Different studies have indicated that some cancer treatments cause highly variable DNA damage among patients, even when the same therapy is applied. This could be partially explained by the fact that different patients exhibit variable DNA repair capacities.[14–16] Therefore, the effect of cancer therapy on DNA integrity depends on individual characteristics. In the present study, we aimed to investigate DNA damage in peripheral blood lymphocytes from breast cancer patients before and after therapy. In addition, we wanted to evaluate whether the genotoxic damage caused by treatments was related to the basal DNA integrity of each patient. Basal DNA damage in patient samples was compared with the values obtained from blood samples of normal donors. Finally, the possible correlation between basal DNA damage and prognostic factors of breast cancer was assessed.

II. MATERIALS AND METHODS

A. Chemicals and Reagents

Unless mentioned in the text, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or from MP Biomedicals (Santa Ana, CA), and they were of the highest purity available.

B. Patients and Donor Samples

The present study was approved by the Centenario Hospital Bioethics Committee (Rosario, Argentina). Blood samples were obtained intravenously and collected in tubes with heparin. Forty-seven breast cancer patients (without family history of breast cancer), from the Mastology Services of the Centenario Hospital and the Provincial Hospital from Rosario (Argentina), and 15 healthy donors (clinically evaluated at Mastology Services) participated in the study. The samples obtained from the cancer patients before tumor surgery and without any treatment; these were considered "pre-treatment" samples. In addition, blood samples were obtained from 24 of the recruited patients at 60 ± 15 days after they finished the first cycle of treatment (chemotherapy and/or radiotherapy) following tumor surgery; these were considered "post-treatment" samples. Both patients and healthy donors signed an informed consent allowing the use of their samples.

C. Separation of Lymphocytes

Lymphocytes were separated by Percoll (Fluka, Steinheim, Germany) density gradients. Briefly, 3.5 ml blood samples were diluted with 0.9% w/v NaCl (Merck, Darmstadt, Germany) and loaded on tubes containing 3 ml of 70% Percoll (density: 1.090 g/ml) and 3 ml of 60% Percoll (density: 1.075 g/ml) and centrifuged for 20 min at 350 × *g*. Cells were collected from the Percoll-blood interphase by aspiration with a Pasteur pipette and washed twice with phosphate-buffered saline (PBS; HyClone, Logan, UT) for 10 min at 200 × *g*. The pellet was then resuspended in PBS. Cells were counted in a hemocytometer chamber, and finally, the concentration was adjusted to 7000 cells per 10 µL. Cell viability was examined using the trypan blue (Mallinckrodt, St. Louis, MO) staining exclusion method.

D. Comet Assay

The comet assay was performed under alkaline conditions according to procedures published elsewhere.[17,18] The assay was carried out in blood lymphocytes from healthy donors and breast cancer patients immediately after the separation of lymphocytes.

A total of three replicate microscope slides were used for each sample. Slides were dipped in a 1% agarose solution (BioRad, Hercules, CA) dissolved in distilled water, and air dried overnight at room temperature. Ten microliters of lymphocyte suspension (7000 cells/10 µL) in PBS was then mixed with 90 µl of 0.5% w/v low-melt agarose solution (Promega, Madison, WI) in PBS, layered onto slides, and allowed to solidify at 4°C. Finally, a layer of 0.5% w/v low-melt agarose solution was added onto the slides, covered with coverslips, and allowed to solidify at 4°C for at least 1 h. After removal of the coverslips, slides were immersed in lysis buffer containing 2.5 mol/l NaCl, 100 mmol/l EDTA (Bio-Rad), 1% v/v Triton X-100 (Promega), 10% v/v DMSO (Riedel de Haën, Seelze, Germany) and 10 mmol/l Tris (Promega), pH 10 for 90 min at 4°C. Slides were then immersed in a horizontal electrophoresis tank filled with alkaline buffer [300 mmol/l NaOH (Cicarelli, Santa Fe, Argentina) and 1 mmol/l EDTA, pH > 13] for 20 min. Afterward, electrophoresis was carried out at 4°C for 20 min at 20 V. Slides were then washed three times with 0.4 mol/l Tris-HCl (Sigma-Aldrich, St. Louis, MO; pH = 7.5), at 4°C for 5 min. After rinsing, slides were fixed with cold ethanol (Merck, Buenos Aires, Argentina) for 10 min, washed with distilled water, and air dried overnight. The slides were stained with 20 µg ml^{-1} ethidium bromide solution (ICN Biomedicals, Aurora, OH), covered with coverslips, and examined at 400× magnification in an epifluorescence microscope (Olympus, Japan) equipped with a digital camera (Olympus D-580). At least 50 randomly selected cells were analyzed for each replicate slide (n = 3) as described below. The evaluation of DNA damage was made either by visual or digital scoring.

E. Visual Scoring

Visual scoring is a subjective method based in visual classification according to the morphological aspect of the comet assay. Comets were classified in five different types (representing increasing amounts of DNA damage), which is a rapid and simple method of analysis with good resolution.[19–21] Types were considered according to tail extent and intensity and head aspect: type 0 (all DNA in the head and no tail: without DNA damage), 1, 2, 3, and 4 (almost all DNA in tail: severely damaged DNA) (Fig. 1). At least 150 comets per sample were classified, and the percentage was calculated for each comet type in the sample. A score for each sample was calculated as the sum of each comet type, previously multiplied by its percentage. Therefore, the score (arbitrary units) ranged between 0 (100% type 0) and 400 (100% type 4).[20]

F. Digital Analysis

The CASP program was used for the image analysis of comets.[22] Digital analysis programs allowed us to obtain different parameters of comets, such as tail length, percentage of tail DNA, percentage of head DNA, and tail moment (product of tail length and percentage of tail DNA), among others.[20] The

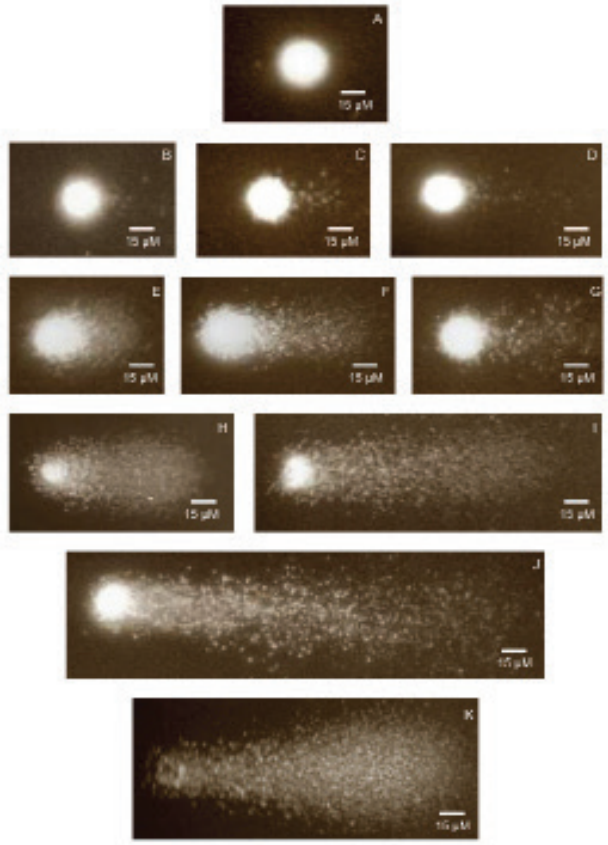

FIG. 1: Visual scoring of DNA damage from 0 to 4 according to appearance of comets. Fluorescence micrographs of comets stained with ethidium bromide and observed in an epifluorescence microscope (400×). (A) Type 0 (absence of DNA damage - comet without tail). (B, C, D) Type 1 (little DNA damage - comet with small tail and big head). (E, F, G) Type 2 (moderate DNA damage - comet with weak tail and big head). (H, I, J) Type 3 (extensive DNA damage - comet with big tail and small head). (K) Type 4 (completely damaged DNA - comet with very small or absent head).

percentages of tail or head DNA are probably the most useful parameters for comparison of results within or between laboratories because they give an immediate, unambiguous, and objective indication of the appearance of comets and are related to the DNA break frequency over a wide range of levels of DNA damage.[20,23] At least 150 comets from each sample were analyzed, and the average percentage of DNA remaining in the comet head (% head DNA) was registered.

G. Tumor Characteristics

Data regarding tumor histology, size, nuclear grade, and detection of lymph-node metastases were obtained from the original pathology reports. Tumors had been diagnosed by senior pathologists using standard criteria for histology and graded using the Scarff–Bloom–Richardson criteria.[24] The immunostaining for estrogen (ER) and progesterone (PR) receptors was performed in fixed sections from tumor tissue using a standard three-layered streptavidin–avidin–biotin horseradish peroxidase method with a mouse anti-human ER primary antibody (1:100 dilution; M7047, DAKO, Ely, Cambridgeshire, UK) or anti-PR antibody 636 (1:100 dilution; M3569, DAKO), respectively, and a biotinylated rabbit anti-mouse secondary antibody (1:350 dilution; E354, DAKO). ER or PR expression was classified positive when staining was observed in more than 10% of cancer cell nuclei. The absence of staining was considered a negative result.[25]

H. Statistical analysis

The correlation analysis between the average % head DNA and prognostic factors, such as the presence of lymph-node metastasis, age of patients, tumor size, and presence of hormone receptors, was carried out by means of contingency tables with proofs based on the Fisher's exact test, using the program GRAPH-PAD INSTAT (GraphPad Software, San Diego, CA).

The Student's t test was used for the statistical comparison between mean values of two different groups. A linear regression analysis between the visual score and the average % head DNA was determined, and between % head DNA before and after treatment for each patient. In addition, the Pearson's correlation (r) coefficient was calculated in both cases. A $p < 0.05$ was considered statistically significant. Results were expressed as media ± SEM.

TABLE 1: Clinical features of the tumors

Characteristic	n	%
Node metastases		
Negative	26	55.3
Positive	21	44.7
Tumor size, cm		
≤2	28	59.5
>2	19	40.5
Histologic grade		
1, 2	18	81.8
3	4	18.2
Nuclear grade		
1, 2	23	76.7
3	7	23.3
Estrogen receptors		
Positive	31	77.5
Negative	9	22.5
Progesterone receptors		
Positive	21	52.5
Negative	19	47.5

Some parameters could not be determined in all the tumors. n and %: number and percentage of tumors with the indicated characteristic.

III. RESULTS

A. Patient Characteristics

The average age was 57.1 ± 1.5 years (range, 29–80 years) for patients and 48.2 ± 3.3 years (range, 24–66 years) for healthy donors. Table 1 shows tumor characteristics from the patients participating in this study. The sum of some of the analyzed parameters differed from the total number of patients because some parameters could not be determined in all 47 patients due to technical problems. Estrogen and progesterone receptors could not be assessed in 7 tumor samples, and histologic and nuclear grades were analyzed only in 22 and 30 tumor samples, respectively.

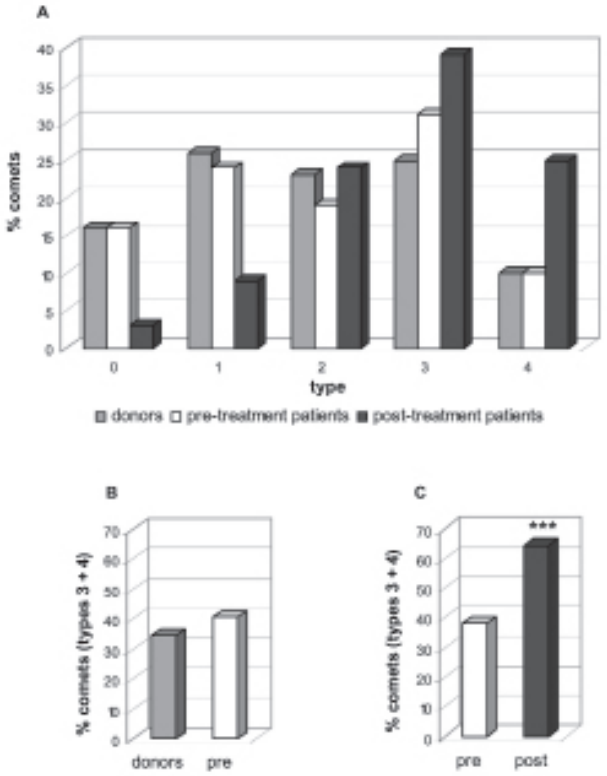

FIG. 2: Distribution of comet types (visual analysis). (A) Distribution of each comet type in lymphocyte samples from donors, pre-treatment patients and post-treatment patients groups. (B) Distribution of comet types (3 + 4) in donors (n = 15) and pre-treatment patients (n = 47) groups. (C) Distribution of comet types (3 + 4) from patients (n = 24) with the comet assay performed before and after treatment. *** $p < 0.001$.

B. Comet Analysis

Cell viability was always higher than 90% for all the lymphocyte samples tested using the comet assay (data not shown). Comets were visually classified under 5 types as indicated in materials and methods (section II.E). Figure 1 shows representative images obtained for each comet type. The average percentage of each comet type did not differ between pre-treatment patients and donors (Fig. 2). Furthermore, there was no difference in the average percentage of each comet type between healthy donors and patients before the treatment (34.6 ± 4.9% vs. 40.7 ± 3.1%, respectively) when only comet types (3 + 4)

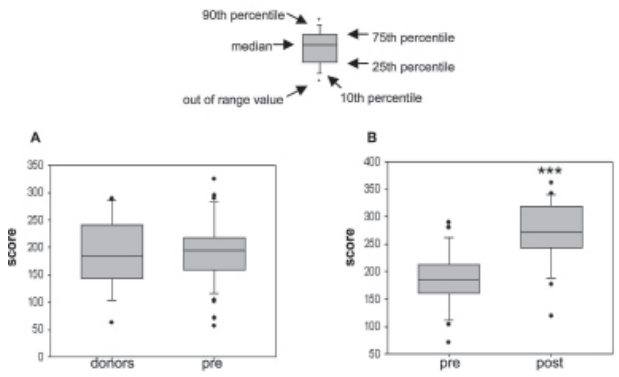

FIG. 3: Score distribution (visual analysis). (A) Score distribution in samples from healthy donors (donors) and pre-treatment cancer patients (pre). (B) Score distribution in samples from breast cancer patients with the comet assay assessed before (pre) and after (post) the cycle of therapy. *** $p < 0.001$.

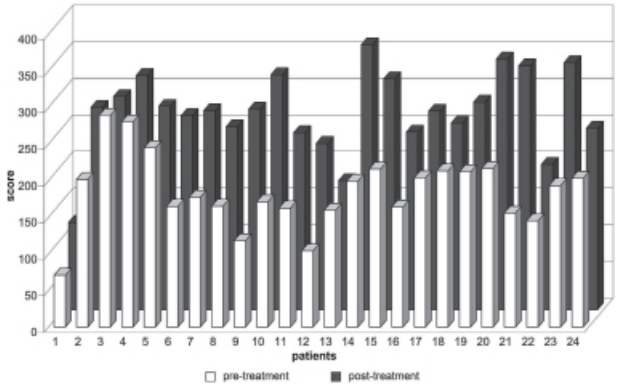

FIG. 4: Scores for the patients before and after treatment. Scores for the samples from the 24 breast cancer patients subjected to the comet assay before (pre-treatment) and after (post-treatment) the first cycle of therapy.

were considered (Fig. 2B). The percentage of comet types (3 + 4) significantly increased in lymphocytes from patients after receiving chemo/radiotherapy, when compared with those found in their respective pre-treatment samples (64.3 ± 4.6% vs. 38.3 ± 3.5%, $p < 0.001$; Fig. 2C).

The visual scores corresponding to normal controls and breast cancer patients before therapy were similar (Fig. 3A). In the same way, scores from post-treatment patients were also significantly higher than the pre-treatment scores ($p < 0.001$; Fig. 3B). It is important to note that in every post-treatment sample the score always increased respect to their value before treatment (Fig. 4).

The parameter chosen for comparative purposes of the digital analysis of comets using the CASP program was the average % head DNA. In agreement with results of visual analysis, there were no differences between the values of the % head DNA for samples of healthy donors and pre-treatment patients (64.0 ± 3.7% vs. 59.7 ± 2.3%, respectively, Fig. 5A). As expected, the % head DNA from post-treatment samples was significantly lower than the pre-treatment value (38.9 ± 3.2% vs. 62.0 ± 2.6%, respectively, $p < 0.001$, Fig. 5B), indicating a marked increase in DNA damage. Data obtained from different groups using visual or digital parameters of DNA damage, were similar. A correlation analysis between comet results from patients before and after treatment indicated an extremely significant correlation ($r = 0.64$, $p = 0.001$).

A value of 65% for % head DNA was chosen and used to classify the level of DNA damage in the pre-treatment samples as low-to-medium (% head DNA ≥ 65%) or high (% head DNA < 65%). Samples were grouped according to this classification to analyze the association of the level of DNA damage in pre-treatment patients with age (< 50 years or ≥ 50 years), the presence of lymph-node metastases, the tumor size (< 2 cm or ≥ 2 cm), the histological grade (1–2 or 3), the nuclear grade (1–2 or 3), and the presence or absence of steroid receptors. As shown in Table 2, no significant associations were found between the level of DNA damage and these characteristics.

Results from cancer patients (with the comet assay performed before and after treatment) were divided according to their pre-treatment level of DNA damage (low-to-medium or high). The average % head DNA values after treatment from each group were then compared. In patients with a high level of DNA damage before therapy, the average % head DNA after treatment was 30.3 ± 3.1% and was significantly lower than the value from post-treatment samples of patients who presented a low-to-medium level of DNA damage previous to treatment (49.2 ± 4.4%; $p < 0.01$; Table 3).

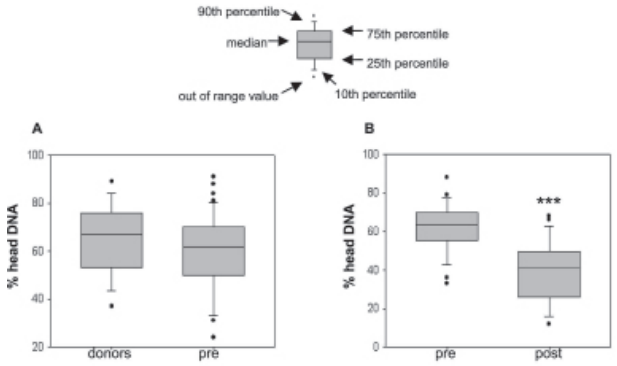

FIG. 5: Distribution of the average percentage of DNA remaining in the comet head (% head DNA; digital analysis). (A) Values of the average % of head DNA in the samples from healthy donors (donors) and pre-treatment patients (pre). (B) Values of the average % of head DNA in the samples from the 24 patients obtained before (pre) and after (post) treatment. *** $p < 0.001$.

C. Visual versus Digital Analysis

The results of both methods used for assessing comets were analyzed by linear regression. Statistically significant correlations between the results of both methods were obtained for the samples of healthy donors ($r = 0.9525$, $p < 0.001$), cancer patients without treatment ($r = 0.9847$, $p < 0.001$), and cancer patients after therapy ($r = 0.9333$, $p < 0.001$). The results indicate that these types of analysis are equally useful in assessing DNA damage.

IV. DISCUSSION

Better therapies have made major contributions to improving survival in cancer, but they have meant exposure of patients to very high doses of ionizing radiation and combinations of high-dose chemotherapy.[1] The risk-benefit equation for a cancer patient often determines the appropriate use of treatment despite acknowledged side effects.[4] In addition, the difference between the amount of drug needed to induce successful tumoricidal action and the amount needed to induce toxicity in the host is small and depends on individual characteristics.[8] Because radiation and most of the drugs applied in breast cancer treatment affect DNA, DNA damage must be considered as a plausible therapy side effect.[15] It has been reported that the comet assay, using peripheral blood lymphocytes, is sensitive enough to detect DNA damage as a result of the cancer treatments because lymphocytes are also affected by the treatments.[14,26]

TABLE 2: Association between DNA damage in the pre-treatment patient's samples and tumor characteristics

Characteristic	n	% head DNA		p
		≥ 65 %	< 65 %	
Node metastases	44			1.000
Negative		9	14	
Positive		9	12	
Age, yr	47			0.122
≤50		10	7	
>50		10	20	
Tumor size, cm	43			0.2194
>2		5	12	
≤2		13	13	
Histologic grade	22			0.1150
1, 2		9	9	
3		0	4	
Nuclear grade	30			1.0000
1, 2		8	15	
3		2	5	
Estrogen receptors	40			0.7053
Positive		13	18	
Negative		5	4	
Progesterone receptors	40			0.3375
Positive		7	14	
Negative		10	9	

n: number of data known for the indicated characteristic. ≥65 % or <65 %: number of samples from pre-treatment patients with low-to-medium or high level of DNA damage (% head DNA ≥65 % or <65 %, respectively).

TABLE 3: Comparison of the average % head DNA after treatment according to the cancer patient's pre-treatment level of DNA damage

Sample	% head DNA (mean ± SEM)		
	≥ 65 % (n = 11)	< 65 % (n = 13)	
Pre-treatment (n =24)[a]	72.3 ± 2.1	53.4 ± 2.7	
Post-treatment	49.2 ± 4.4	30.3 ± 3.1	[b]p = 0.002

Pre-treatment cancer patients were divided into two groups according to the level of DNA damage. Results are expressed as means ± standard error of the mean (SEM), n: number of patients within each subgroup.

[a]Only patients with the comet assay performed before and after treatment. [b]The mean post-treatment values of % head DNA from each group were significantly different ($p < 0.01$).

It has been suggested that DNA structure in normal cells from cancer patients is more altered than in cells from healthy individuals.[11] In this regard, some studies have reported that DNA of peripheral blood lymphocytes from sporadic breast cancer patients was more damaged than that from healthy donors.[15,27–29] In contrast, other researchers have not found significant differences between the basal damage in lymphocytes from patients with sporadic breast carcinoma and healthy donors.[30–33] In the present study, the comet assay results of samples from healthy donors and pre-treatment cancer patients were not significantly different, either.

Additionally, it has been proposed that many factors such as age, gender, alcohol consumption, excessive exercise and diet, among others, could affect the result of comet assay; however, in most studies, the lack of impact of these factors on the results has been reported.[9,34] DNA damage may also increase with age due to an increased frequency of mutations, resulting in accumulation of damaged and unrepaired DNA and a reduced DNA repair capacity, but many reports do not support this assumption.[9,10] In several studies, the authors did not find an association between age and DNA damage when using the comet assay.[5,28,29,35,36] In the present study, no association between levels of DNA damage and age (> 50 years or ≤ 50 years) was found.

In addition, the results of the present study showed no significant associations in levels of DNA damage with the presence of lymph-node metastases, the tumor size, the histological grade, the nuclear grade, and the presence of steroid receptors in pre-treatment cancer patients.

DNA integrity of peripheral blood lymphocytes in breast carcinoma patients after therapy was also evaluated. A significant increase in DNA damage was observed in all post-treatment samples when compared to pre-treatment samples. This finding is in agreement with previous reports,[5,15,27,37] which have indicated that the administration of antineoplastic drugs and radiotherapy are accompanied by significant DNA damage to nucleated blood cells. The previous data and the present results support the usefulness of the comet assay as a sensitive technique to evaluate damage caused by radiotherapy or chemotherapy.

The results of this study reveal a significant correlation between the DNA integrity before and after cancer treatment. Cancer patients with high DNA damage before treatment showed significantly lower values of % of head DNA after therapy than patients with low-to-medium DNA damage pre-treatment. Thus, it can be inferred that DNA damage caused by cancer treatments is associated with the basal DNA integrity of each patient. Based on this idea, basal DNA integrity of a patient's lymphocytes could be assessed before anticancer therapy to help avoid an excessive collateral damage associated with treatment. Therefore, the comet assay could contribute to the treatment decisions by considering the patient's basal DNA damage before initiating therapy.

On the other hand, and in agreement with previous data,[38,39] visual and digital analysis revealed

highly significant correlations for all sample values and showed to be equally useful to compare the level of DNA damage between different groups. However, the visual method can be influenced by the observer experience; thus, it is more subjective.[40,41] Although image analysis provides more objective information, Duez et al. reported 4% variability among operators.[42] Thus, and as in most analytical techniques, it is important to maintain the same operator throughout the study to avoid these variations.

In conclusion, the efficiency of anticancer therapy is limited by the associated adverse side effects having an impact on normal tissues, including several non-malignant conditions and a potential risk for developing secondary malignant neoplasms. In addition, the magnitude of side effects is different in each patient despite receiving the same treatment. Thus, different approaches for evaluating individual risk before treatment could be very helpful in diminishing the side effects of therapy. One approach could be to assess basal DNA integrity in peripheral lymphocytes in each patient before therapy using the comet assay. Compared to other tests for genotoxic damage, the principal advantages of the comet assay include its demonstrated sensitivity for detecting DNA damage at a very low degree, the requirement for a small number of cells per sample, its flexibility, low cost, and the relatively short time needed to complete a study.[43–45]

The data collected in the present study suggest that the comet assay could contribute to treatment decisions by considering the patient's basal DNA damage before therapy. At present, therapy decisions are largely based on prognostic factors, such as the presence of node metastases, among others. However, treatment side effects vary among patients and are hard to predict. Therefore, adding the comet assay to regimen of clinical exams carried out before treatment could contribute to determining the best possible course of treatment for each patient, in a complementary way to the prognostic factors, to avoid the risk of excessive DNA damage caused by treatments. Therefore, side effects from therapy could be reduced, and patients could benefit. Using the comet assay to inform treatment decisions, patients with low basal DNA integrity could receive less aggressive treatments, could avoid the combination of chemotherapy and radiotherapy, or could receive reduced doses or frequency of these therapies. However, these approaches remain to be investigated.

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