Original Article
DNA repair in Etoposide-induced DNA damage in lymphocytes of breast cancer patients and healthy women

Ana Claudia Teixeira[1], Raquel Alves dos Santos[1], Aline Poersch[1], Helio Humberto Angotti Carrara[2], Jurandyr Moreira de Andrade[2], Catarina Satie Takahashi[1,3]

[1]Department of Genetics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14040-900 Ribeirão Preto, SP, Brazil. [2]Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14040-900 Ribeirão Preto, SP, Brazil. [3]Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14040-901 Ribeirão Preto, SP, Brazil.

Abstract: The present study evaluated the basal DNA damage and the cellular response to this damage induced by *in vitro* administration of Etoposide in lymphocytes donated by twenty untreated breast cancer (BC) patients and twenty age-matched healthy women. Micronucleus (MN) and alkaline Comet assays were performed in cultured peripheral blood lymphocytes (PBL) according to a standard protocol for *in vitro* treatment with various concentrations of Etoposide or a control. For the Comet Assay, three samples of cells were collected: T_0 (immediately preceding treatment of the cultures), T_1 (immediately after completion of the treatment) and T_2 (four hours after completion of the treatment). MN frequency in the BC group treated with 25 µM Etoposide (19.1 ± 7.35) was significantly higher than the control (10.9 ± 9.87) group. In the alkaline Comet Assay, both the BC group and the healthy women showed the ability to repair Etoposide-induced DNA damage within 4 hours of reincubation.

Key words: Micronucleus Test, Comet Assay, breast cancer, DNA repair, etoposide

Introduction

Despite years of intensive study and substantial progress in understanding breast cancer (BC) susceptibility, this disease remains a leading cause of death in women and thus represents the major women's health issue in most industrialized countries [1,2].

For the development of preventive strategies, it is important to identify both inherent and acquired factors that influence individual risk for developing cancer [3]. A potentially important source of inherent genetic susceptibility to development of cancer is interindividual variability in the DNA repair capacity within the human population. Common genetic polymorphisms in the DNA repair genes may alter protein function and thus an individual's capacity to repair damaged DNA. Such deficiencies in DNA repair capacity may in turn lead to genetic instability and carcinogenesis [4].

The DNA repair pathway is essential for maintaining genomic stability. Deficiencies in the DNA repair system are likely to cause chromosomal aberrations and micronuclei, which can lead to cell malfunctioning, cell death and tumorigenesis. Low-penetrance cancer susceptibility genes, including DNA repair genes, may be associated with a large number of breast cancer cases. To address this possibility, a great number of studies have screened DNA repair genes for the presence of polymorphic alleles, and have found a variety

of point mutations associated with cancer risk [5]. Mammalian cells are constantly exposed to a wide variety of genotoxic agents from both endogenous and exogenous sources. The response of the cell to DNA damage and its ability to maintain genomic stability by DNA repair are crucial for preventing the initiation and progression of cancer. Genetic variability in DNA repair genes may affect the final product of their expression, and consequently may influence individual susceptibility to tumorigenesis [6].

The study of DNA damage at the chromosomal level is essential to genetic toxicology because chromosomal mutation can be an early event in carcinogenesis. The Micronucleus Test (MN) and the Comet Assay are among the methods most frequently used to assess DNA damage; here, we combined these two methods to investigate damage caused by Etoposide, an inhibitor of topoisomerase II and a very powerful staple of chemotherapy. Topoisomerase II-inhibiting drugs are the backbone of many chemotherapeutic strategies [7,8].

Micronucleus formation is the result of the loss of either the whole chromosome or acentric fragments of the chromosome from the nucleus as a consequence of structural chromosomal damage or disturbance of the mitotic apparatus. Therefore, DNA damage in the form of micronuclei, as measured by the MN Test, is only observed in cells that have completed a cell division cycle [9].

The Comet Assay is a fast, simple and sensitive method for the quantification of genetic damage in a small number of cells. This method is particularly useful in the detection of intercellular differences in that it allows for the possible detection of DNA damage in individual cells, as well as DNA damage and repair in virtually any eukaryotic cell type where it is possible to obtain a cell suspension with even a small number of cells per sample [10-16]. For these reasons, the Comet Assay has been used to evaluate DNA damage induced by physical and chemical agents in numerous studies involving environmental monitoring and medical research [14].

The present work aimed to evaluate and compare the basal damage and sensitivity of DNA and the repair response after *in vitro* Etoposide-induced DNA damage in lymphocytes in BC patients and healthy women.

Materials and methods

Subjects and samples

Blood samples were obtained from 20 untreated women with ductal breast carcinoma (mean age 49.9) and 20 healthy control women (mean age 45.3); all of the samples were gathered at The University Hospital (Ribeirão Preto, São Paulo, Brazil) from the Department of Gynecology and Obstetrics. This investigation was approved by the National Ethics Committee (CONEP N° 9238/2006) and is in accordance with ethical standard procedures. Before entering the study, all subjects were informed about the objectives and experimental details of this research. Informed consent for voluntary participation was given by all participants.

Blood sampling, cell culture and treatments

Approximately 10 mL of peripheral blood were taken from BC patients and healthy control women by venous puncture. The blood was drawn into heparinized vacuntainer® tubes and kept at 4°C in the dark until use.

Cell cultures were prepared by adding isolated lymphocytes with plasma to 5 mL of complete medium containing 78% RPMI (Sigma - Aldrich Co., USA), 20% inactivated fetal bovine serum (Gibco - Invitrogen, Denmark), antibiotics (penicillin and streptomycin) and 2% phytohemagglutinin (Gibco - Invitrogen, Denmark).

In the Micronucleus test, cultures were incubated at 37°C for 44 hours, after which treatments with Etoposide at 5 μM, 10 μM and 25 μM were performed for 1 hour in serum-free medium. At the end of the treatments, cultures were washed twice with RPMI medium and reincubated with complete medium supplemented with Cytochalasin B (5 μg/mL) for 28 more hours to block cytokinesis and induce binucleated cells.

In the Comet Assay, cultures were incubated at 37°C for 24 hours, after which treatments with Etoposide at 5 μM, 10 μM and 25 μM were performed for 1 hour in serum-free medium. At the end of the treatments, cultures were washed twice with RPMI medium and reincubated with complete medium for 4 hours. In this test, DNA repair capability was evaluated by harvesting cells immediately before the cell culture treatment (T_0), immedi-

ately after treatment (T_1) and four hours after the completion of the treatment (T_2).

Micronucleus Test

Binucleated cells for micronuclei analysis were obtained according to Fenech and Morley [17] as described above.

Briefly, after the treatments were completed, the cells were subjected to a mild hypotonic treatment (1% sodium citrate), fixed twice with methanol:acetic acid (2:1), and then smeared onto a pre-cleaned microscope slide and air-dried. The slides were stained with 5% Giemsa diluted in phosphate buffer (0.06 M Na_2HPO_4 and 0.06M KH_2PO_4, pH 6.8) for five minutes, washed with distilled water, air-dried and kept until microscopic analysis. The frequency of MN was determined by a blind test in 1000 binucleated cells with the cytoplasm well preserved using a Zeiss (Germany) microscope. The criteria for the identification of MN were according to Fenech [18] and Titenko-Holland et al. [19].

The Nuclear Division Index (NDI) was determined by a blind test in 1000 cells. Cells with well preserved cytoplasm, containing 1 to 4 nuclei, were scored using a Zeiss (Germany) microscope. The NDI was calculated according to Eastmond and Tucker (1989) [20] using the following formula:

$$NDI = [M1 + 2(M2) + 3(M3) + 4(M4)]/ N$$

where M1 - M4 are the numbers of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of viable cells.

Comet Assay

The Comet Assay was performed under alkaline conditions, as described by Singh *et al*. [21] with modifications. Briefly, 300 μL of cellular suspension was centrifuged for 5 min at 500 rpm. The pellet was homogenized in 100 μL of low melting point agarose (0.5%) at 37°C. The cell mixture was allowed to set on ice for 5 min, and than spread on micro-scope slides and the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10.0) overnight at 4°C. The slides were then placed into electrophoresis buffer (0.3 M NaOH and 1 mM Na_2EDTA, pH > 13) for 20

min at 4°C. Electrophoresis was performed at 25 V (1V/cm) and 300 mA for 20 min at 4°C. The slides were washed with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min and then air dried. Slides were stained with 20 μg/mL of ethidium bromide immediately before analysis.

DNA damage was determined in 100 nucleoids in a blind test using a fluorescent microscope (Zeiss, Germany) equipped with an excitation filter of 515-560 nm and barrier filter of 590 nm (40x objective). The following visual score based on the extent of migration was used: 0 (< 5% of migrated DNA), 1 (5 – 20% of migrated DNA), 2 (20 – 40% of migrated DNA), 3 (40 - 95% of migrated DNA) and 4 (> 95% of migrated DNA). To facilitate management of the data, an average of DNA migration (DNA damage) was calculated as follows: (n° of cells with score 1) x 1 + (n° of cells with score 2) x 2 + (n° of cells with score 3) x 3 + (n° of cells with score 4) x 4 / 100.

Statistical analysis

The Mann-Whitney statistical test was applied to compare micronucleus frequency, NDI and DNA damage between BC patients and healthy women. The statistical test was performed using SigmaStat 3.0 (Jandel Scientific) and differences with *P* values lower than 0.05 were considered statistically significant.

Results

Table 1 compares BC patients and healthy women according their age, smoking habits, menopause status, oral contraceptive and hormone replacement therapy (HRT). No statistical differences were observed between BC group and healthy women according to these factors.

Figure 1 shows that the different treatments performed (control and Etoposide at 5, 10 and 25 µM) did not significantly reduce the NDI in patients and healthy control women, or even when both groups were compared to each other.

The frequency of MNs in the cultures from the BC group treated with 25 µM Etoposide (19.1 ± 7.35) was significantly higher than that of the control (10.9 ± 9.87) group (*P* < 0.05) (**Figure 2**). Although Etoposide has a trend of

Table 1. Characteristics of breast cancer patients and healthy women

Factor	BC Patients (n = 20)		Healthy Women (n = 20)		P
	N	%	N	%	
Age					
≤ 45 years	4	20.0	9	45.0	0.17
> 45 years	16	80.0	11	55.0	
Smoking					
No	17	85.0	16	80.0	0.67
Yes	3	15.0	4	20.0	
Menopause status					
Yes	9	45.0	11	57.9	0.62
No	11	55.0	8	42.1	
NI	-	-	1	-	
Hormones					
Oral contraceptive					
Yes	2	10.0	3	15.0	0.63
No	18	90.0	17	85.0	
HRT					
Yes	3	15.0	4	20.0	0.67
No	17	85.0	16	80.0	

P-value from X^2 –test; NI: Not informed; BC: Breast cancer patients; HRT: Hormone Replacement Therapy.

increasing MN frequency in the BC group, a significant difference between the BC group and healthy women was observed only at the highest concentration (25 µM) (**Figure 2**).

Figure 3 shows the results of the Comet Assay. In T_1, Etoposide (5, 10 and 25 µM) significantly increased the level of DNA damage in both BC patients and healthy women. However, differrences between BC patients and healthy women were not observed when compared within the same time point (T_0 vs. T_0; T_1 vs. T_1; T_2 vs. T_2) (**Figure 3**).

There was a difference observed when T_0 was compared with T_1, and T_1 with T_2, in both groups (BC patients and healthy women). However, there was no significant difference between T_0 and T_2 for all of the Etoposide treatment concentrations in BC patients and healthy women (**Figure 3**).

Discussion

In recent years, many studies have provided evidence that an association exists between breast cancer of both sporadic and familial origin and DNA repair, suggesting that a reduction in the DNA repair capability of an individual could be genetically determined and confer BC susceptibility [22, 23, 5]. The response of the cell to genetic injury and its ability to maintain genomic stability by means of a variety of DNA repair mechanisms are essential in preventing tumor initiation and progression and the mechanisms involved in DNA double strand break (DSB) repair are of particular etiological importance during breast tumorigenesis [24, 25].

One of the objectives of this work was to evaluate the basal DNA damage and the DNA repair capability in lymphocytes of BC patients
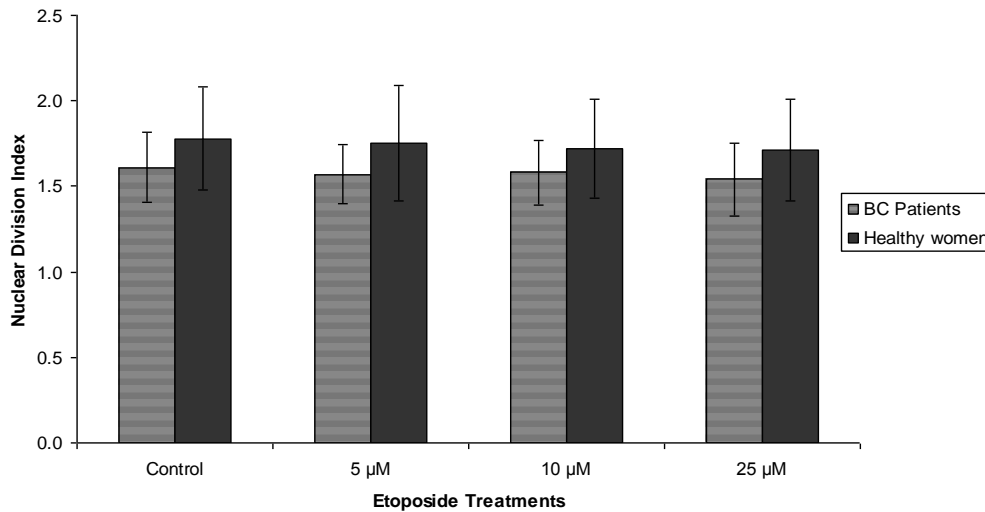
Figure 1. Effect of *in vitro* treatment of Etoposide in Nuclear Division Index (NDI) in BC patients and healthy women.
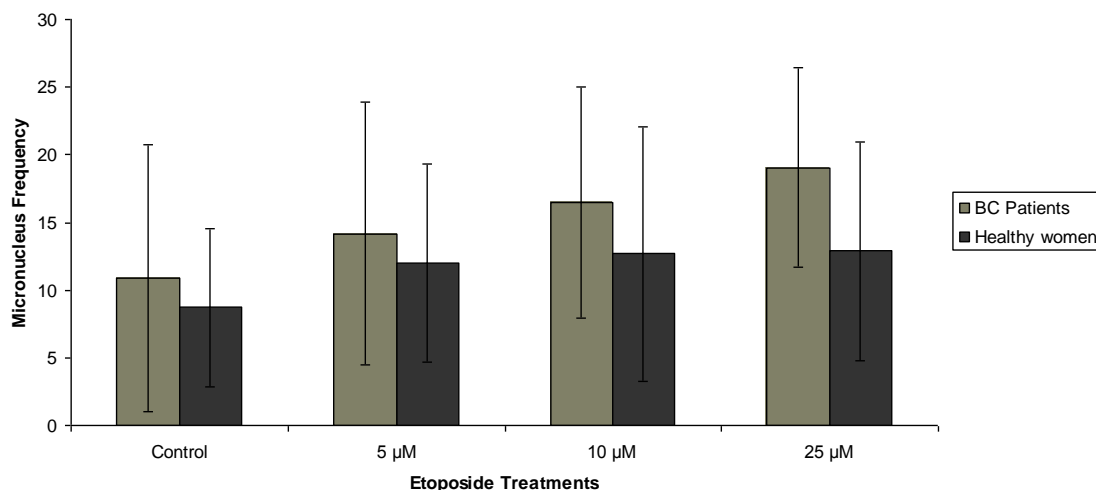

Figure 2. Effect of *in vitro* treatment of Etoposide in Micronucleus Frequency in BC patients and healthy women

and healthy women after Etoposide-induced DNA damage, *in vitro*. These parameters were assessed using the Nuclear Division Index (NDI), MN frequency and the Comet Assay.

There were no significant differences observed between the BC group and healthy women when both groups were characterized according their age, smoking habits, menopause status, oral contraceptive and hormone replacement therapy, confirming the homogeneity of the sample in this study.

Significant differences in the NDI were observed between the different treatments relative to the negative control in cultures derived from BC patients, as well as when same treatment was compared between the lymphocytes from the two groups (patients and healthy control women). These results suggest that the different concentrations of Etoposide did not induce cytotoxic events, and therefore, it does not interfere in the cell cycle kinetics. Lebailly *et al* [26] showed that Etoposide was
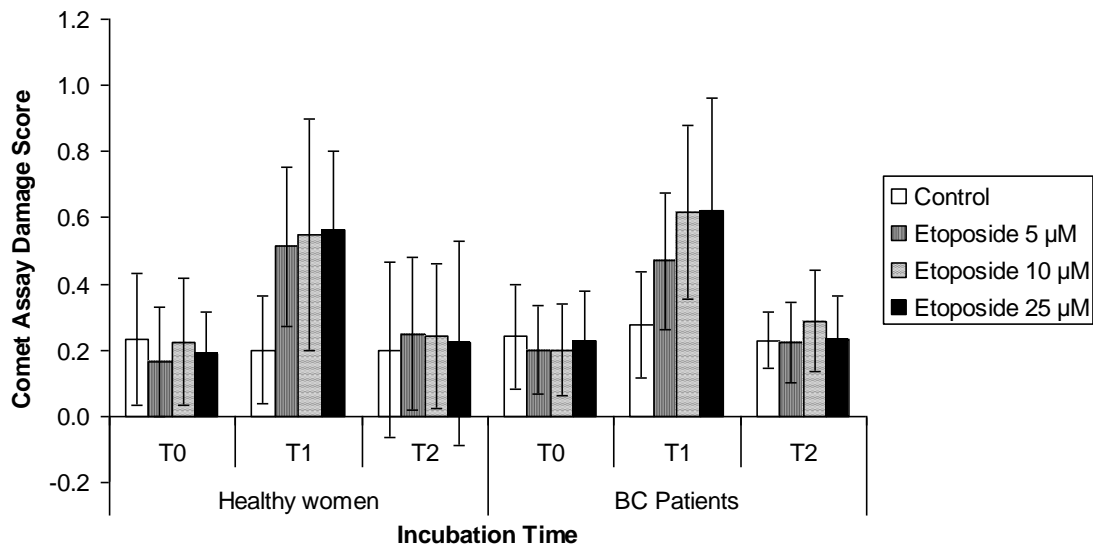
Figure 3. DNA repair capability peripheral lymphocytes of BC patients and healthy women after *in vitro* treatment with Etoposide. T_0: before treatments; T_1: immediately after treatments; T_2: 4 hours after treatments.

weakly toxic to human peripheral blood lymphocytes after treatment for 1 hour and induced a dose-dependent DNA damage at different concentrations.

We did not observe significant differences in frequency of basal levels of MN between patients and healthy controls. The investigation of basal levels of DNA damage in peripheral lymphocytes of untreated cancer subjects has been previously reported. Baseline levels of DNA damage were significantly higher in bladder cancer patients than in controls [27], and the frequency of MN in stimulated peripheral blood cells from an untreated leukemia population was significantly greater than that of the control group [28]. Similar results were found by Lou et al. [29], who simultaneously investigated both baseline and ionizing radiation-induced (IR) genetic damage in peripheral lymphocytes from 36 cancer patients using MN and Comet assays. They found that both spontaneous and IR-induced genetic damage were higher in patients than in controls. Varga et al. [30] demonstrated in a case-control study, the increase in the frequency of MNs observed in lymphocytes of patients with sporadic BC may be due to a defect in DNA repair, resulting in a cellular phenotype of increased sensitivity to mutagenic agents.

According to Bromberg et al. [31], Etoposide treatment generates a high ratio of single-stranded to double-stranded DNA breaks at low drug concentrations. This type of DNA damage are primary lesions in DNA molecule that can account to more drastic lesions such as chromosomal damage generating the instability commonly observed in solid tumors [29]. In the present study we investigated the sensibility and the DNA repair capacity of lymphocytes from breast cancer patients compared to healthy women.

The results presented here demonstrated a statistically significant difference between the BC group and healthy women at the highest concentration of Etoposide (25 µM). The difference in MN induction at the different concentrations could be due to the possibility that 5 and 10 µM Etoposide induced other forms of genetic damage, as was reported by Vukicevic et al. [32], which did not result in the formation of MNs. Furthermore, the data obtained with the MN Test in this study corroborate the *in vivo* study performed by Turner et al. [33], in which the induction of DNA damage was similarly only verified at the highest concentration of Etoposide, suggesting that the deleterious effects of this drug is dose-dependent. In other words, small concentrations of Etoposide may induce only small

amounts of DNA damage, as observed by Lebailly *et al*. [26]. Another factor that could contribute to the observed results is the finding that when Etoposide is removed from the culture media, damage to the DNA is quickly repaired [34, 35]. This suggests that the damage induced by 5 and 10 µM concentrations of Etoposide may have been repaired during the period of action required for Cytochalasin B (28 hours). The results of the Comet Assay appear to confirm this hypothesis, as this assay was able to ascertain differences between control and treatments with all concentrations of Etoposide in BC patients and healthy women. Is well known that the Comet Assay detects genomic lesions that are under the action of DNA repair machinery, and is therefore more sensitive than the MN assay, which detects only the consequence of unrepaired lesions [36].

The elevation in DNA damage observed at a concentration of 25 µM Etoposide in the MN Test could be explained by the fact that micronuclei are features of irreversible DNA loss that are induced in a dose-dependent fashion. This allows them to be quantified as a mutation index [37]. According to Varga *et al*. [30], there are two possible explanations that should be considered when using such a measure. The first is that the lymphocytes of patients represent a generally more sensitive heterogeneous subpopulation of cells when compared to those of healthy individuals. The second possibility is that these same lymphocytes have different properties specifically regarding the induction of DNA damage and its repair.

Nowadays, there is great interest in the application of the Comet Assay because it is a simple method that has been widely used to detect the degree of DNA damage in individual cells in genotoxicity tests and biomonitoring under several conditions [21]. However, there is some conflict in the results obtained with the Comet Assay in studies regarding BC patients. While many researchers have found an increase in sensitivity to double-strand breaks and a low repair capacity in the peripheral blood lymphocytes of these patients [38], others did not report such differences in *in vitro* studies in which the lymphocytes were exposed to genotoxic chemical agents [39].

It was observed a significant difference between negative control and Etoposide treatments (5, 10 and 25 µM), in both studied groups (BC patients and healthy control women) after 1 hour of treatment (T_1), when the DNA damage levels of both groups underwent a gradual increase in score in a dose-dependent manner [26]. Thus, even small amounts of Etoposide-induced DNA damage could be detected by the Comet Assay due to the high sensitivity of this test.

After 4 hours of incubation (T_2), the levels of DNA damage in both studied groups were lower and closer to the level of basal damage observed at T_0, indicating that repair of this DNA damage may have occurred. According Wozniak and Ross [34] and Van Maanen *et al*. [35], the DNA damage induced by Etoposide is quickly repaired when the drug is removed from the culture medium, an effect that may have contributed to the damage levels observed in T_2 being close to these observed in T_0 in both the BC patient group and healthy women.

In conclusion, these results demonstrate that was not observed difference in the DNA repair capacity of the lymphocytes from BC patients and healthy women after Etoposide-induced DNA damage.

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