

Genotoxic and Cytotoxic Damage by Cyclophosphamide and Adriamycin as a Response to Treatment in Breast Cancer Patients: Pilot Study

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Abstract

The aim of this study was to evaluate the genotoxicity induced by cyclophosphamide-adriamycin treatment in breast cancer patients through the frequencies of Sister Chromatid Exchange (SCE), Replication Index (RI), Mitotic Index (MI) and Cell Proliferation Index (CPI) and to study the possible association between biomarkers of genotoxicity and the early response to treatment. The frequencies were obtained before and immediately after therapy from 17 patients with breast cancer ($p < 0.001$). Response to treatment was assessed after two years resulting in 12 patients in a state of remission. MI and CPI had high values after treatment in women with active cancers compared to those in a state of remission, however there were not significant differences. Conclusions: It is possible that MI y CPI biomarkers can serve as indicators for early assessment of treatment with cyclophosphamide-adriamycin. It should be noted that these are preliminary results and further study is necessary.

Keywords

Sister Chromatid Exchange, Genotoxicity Biomarkers, Breast Cancer, Cyclophosphamide, Adriamycin

1. Introduction

Breast cancer is a serious threat for women's health; more than 10 million women are diagnosed each year in the

world with this malignancy. In Mexico, with a population of over 100 million, between 2 and 4 women per 100,000 die from breast cancer; an increment of 16,500 new cases are estimated for 2020 [1] [2]. Therapy with cyclophosphamide and adriamycin as chemotherapeutic agents is one of the most widely used to treat breast cancer. These drugs exert their effect by disrupting DNA, which leads to cell death, resulting in the control of the disease and on the other hand, in a lot of secondary effects [3] [4]. Cyclophosphamide is an antineoplastic drug with immunosuppressive properties. It belongs to the family of the alkylating agents and it is considered one of the most potent drugs ever synthesized. It needs to be activated by the hepatic microsomal enzyme system in order to be cytotoxic. During that metabolic activation, involving the alkyl and nucleophilic sites of DNA, adducts are formed [5]. Alkylation products are important in the biological effects caused by this and other alkylating agents. It is a strong bone marrow depressant, which is its biggest toxic effect: it leads to a dose related suppression of myelopoiesis, directly attacking the bone marrow. Lymphoid proliferative cells are destroyed, but some resting cells can also be attacked [6]. Adriamycin, also called doxorubicin, belongs to the anthracycline family. These drugs are among the more used cytotoxic drugs against cancer and it has become the main treatment of breast cancer [6] [7]. The adverse effects include: bone marrow depression, related cardiac toxicity, which increases with the dose accumulation and severe or total alopecia at standard dose [6].

On the other hand, it has been shown that cyclophosphamide is an excellent inducer of Sister Chromatid Exchanges (SCE), which is a sensitive biomarker for detecting the mutagenic and oncogenic potentiality of various agents [8]. It has also been reported that the Mitotic Index (MI) and Replication Index (RI), together with other biological markers as Cell Proliferation Index (CPI) are suitable indicators of cell proliferation, especially in genotoxicity studies, because of their sensitivity to determine the cytotoxic and cytostatic action of various environmental pollutants and therapeutic agents [9] [10].

For the evaluation of response to this treatment, a clinical assessment is performed after two to three cycles, using the system of the World Health Organization (WHO) or the RECIST system [11]. Nowadays, it is impossible to know whether the therapy will be completely effective for the patient after the first application, the evaluation is made usually when half of the treatment has been administrated, usually after a period of 4 to 5 months. At this point patients not responding to the treatment will be detected, along with patients in whom the toxicity was too high to continue the scheme. Jones *et al.* [12] studied a group of 55 women with breast cancer treated with adriamycin-cyclophosphamide, it was found that 83% had a favorable response to treatment, while the rest did not show an overall improvement after four cycles. Finally, this study aimed to determine the genotoxicity induced by adriamycin-cyclophosphamide and the possible association between the frequencies of SCE, RI, MI and CPI and the early response to treatment in breast cancer patients.

2. Materials and Methods

2.1. Studied Group

The patient group was formed by 17 women with breast cancer (mean age 47 years) from the chemotherapy service of the ISSEMyM State Oncology Center (Toluca, México). The study excluded patients who had had previous cancer treatment. Written informed consent was obtained from each participant according to the official Mexican regulations in the field of health research [13]. The authors certify that the study was approved by the Bioethics Committee of the ISSEMyM State Oncology Center and that all experiments were done under full compliance with all government policies and the Helsinki Declaration [14].

2.2. Chemotherapy

Patients were treated with adriamycin (60 mg/m²) and cyclophosphamide (600 mg/m²) on Day 21 of the cycle. The scheme was repeated during 8 cycles in patients under adjuvant chemotherapy and 4 cycles for those under neoadjuvant chemotherapy.

2.3. Blood Sample

Two blood samples were taken by venous puncture in the arm, in a heparinized vacutainer tube from each patient. The first sample (3 mL) was taken right before starting the therapy, to determine the basal rate. Subsequently, the second sample of 7 mL was obtained 5 minutes after the end of the first dose. All samples were stored at room temperature until the time of the test.

2.4. Lymphocyte Culture

The lymphocyte culture medium consisted of 5 mL of RPMI-1640 (Sigma, USA), 0.25 mL of phytohemagglutinin (Sigma, USA), 0.4 mL of penicillin-streptomycin antibiotic (Microlab, Mexico) and 0.25 mL of BrdU (bromodeoxyuridine (10 µg/mL; Sigma, USA). Whole blood (12 to 18 drops) was added to the culture medium and incubated for 72 hours at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in 5% CO_2 atmosphere.

2.5. Determination of Biomarkers before Treatment

After 72 hours, 0.3 mL of colchicine (10 µg/mL; Sigma, USA) was added to the culture flask and incubated for 1 hour. The rest of the harvest was performed using the Moorhead method [15]. Finally, the slides were stained according to the modified technique of Perry & Wolf [16], and coded for blind analysis.

2.6. Determination of Biomarkers after Treatment

Considering that a high cell death occurs because of the treatment, we added 24 hours at the very beginning of the incubation, in order to obtain an acceptable cell number to examine SCE, RI, MI and CPI. After these 24 incubating hours, 0.25 mL of BrdU (10 µg/mL) was added to the lymphocyte culture, the incubation continued for 72 more hours (the total time of culture was 96 hours). Harvest of cells was then performed by adding 0.3 mL of colchicine to the culture flask (Sigma, USA, 10 µg/mL) and incubated for 3.5 hours (37°C , 5% CO_2).

2.7. Analysis of Biomarkers

For SCE, 30 metaphases in the second cycle of replication were analyzed. Terminal SCEs were counted as an event and intercalary ones as two. For RI, 100 consecutive metaphases were counted by separating the first, second and third cycle of replication. The formula proposed by Krishna [17] was applied: $\text{RI} = [1(\text{M1}) + 2(\text{M2}) + 3(\text{M3})]/100$, where M1, M2 and M3 represent the number of metaphases in the first, second and third replication cycles respectively. For MI, the number of metaphase cells found in 2000 successive nuclei was evaluated with the following formula: $\text{MI} = (\text{number of metaphases} \times 100)/2000$ transformed nuclei. For CPI, 2000 nuclei were counted by identifying transformed, necrotic and apoptotic nuclei, according to Fennec [18], and the following formula was applied: $\text{CPI} = (\text{transformed} \times 100 \text{ nuclei})/\text{nuclei counted}$.

2.8. Statistical Analysis

The data was analyzed with the Shapiro-Wilk test. Paired t-Test was applied to SCE, CPI and Wilcoxon test to RI and MI and the treatment response was analyzed by Mann-Whitney U test. The statistical program Sigma Plot for Windows (Version 11.0, Systat Software, Inc.) was used.

3. Results

Samples were collected from 17 breast cancer patients, whose average age was 47 years in a range of 37 - 61 years, they had the disease in Stages IIA (23.5%), IIB (29.4%), IIIA (17.7%), IIIB (17.6%) and two cases were non classifiable (11.8%). For SCE, there was an average of 0.64 ± 0.25 SCE/cell in a range of 0.37 to 1.23 before chemotherapy (bQT); and an average of 1.63 ± 0.90 SCE/cell in a range of 0.57 to 3.4 after chemotherapy (aQT). Paired t-Test showed significant difference ($p = 0.001$) between before and after treatment, all these results are shown in **Table 1**. The analysis of the values of each of the cases showed that 47% of patients exhibited a dramatic increase in SCE bQT (more than 200%) compared with the values of aQT.

For the CPI there was an average value of 63.6 ± 17.7 in the range of 30.4 to 99.9 bQT and 28.3 ± 23.8 in the range of 4.5 to 89.2 aQT. According to the paired t-Test, there were statistically significant differences ($p < 0.001$; **Table 1**) between the values aQt and bQT. Moreover, it was found that approximately 29% ($n = 5$) of the cases presented a markedly decrease in the CPI, 6 times less than the value before treatment (63.3 bQT versus 10.8 aQT).

For RI, there was a decrease of bQT versus aQT results, a median of 1.46 in a range of 1.16 to 1.61 against a median of 1.15 in a range of 0.15 to 1.44 respectively. The Wilcoxon test showed a significant difference ($p < 0.001$), all this results are shown in **Table 2**. The analysis per individual demonstrated that 11.7% of the cases presented a decrease in RI (1.42 in the values of bQT versus 0.22 aQT). In the case of MI, it was obtained a

Table 1. Biomarker results of SCE and CPI values in blood lymphocytes before and after treatment.

Biomarker	Mean Value bQT (\pm SD)	Mean Value aQT (\pm SD)
SCE	^a 0.64 \pm 0.25	1.63 \pm 0.90
CPI	^a 63.6 \pm 17.7	28.3 \pm 23.8

^aPaired t-Test for SCE and CPI before vs after chemotherapy, $p < 0.001$.

Table 2. Biomarker results of RI and MI values in blood lymphocytes before and after treatment.

Biomarker	Median bQT	Median aQT
RI	1.46 ^a	1.15
MI	1.3 ^a	0.38

^aWilcoxon test for RI and MI before versus after chemotherapy, $p < 0.001$.

median of 1.3 bQT with a range from 0.15% to 16.6% and a median of 0.38 aQT with an interval of 0.05% to 16.2%. Wilcoxon test showed that the decrease was significant ($p < 0.001$); these results are show in **Table 2**. The analysis of MI per individual showed that 34.8% of the cases presented a decrease, 2.1 in the values of bQT versus 0.19 aQT. Analysis of data from all selected biomarkers showed significant difference in all patients when comparing baseline data (bQT) with those values obtained after the first dose of treatment.

In order to find out if any of the biomarkers may be an indicator of early response to treatment, the clinical status of all patients after completion of 8 treatment cycles was documented by reviewing their records. On this basis, those patients who reported an active state of the neoplasm and those in remission were grouped; four patients showed an active state and twelve a state of remission, the difference was not statistically significant (Mann-Whitney U test) between patients with neoplasm ($n = 4$) compared with patients in remission ($n = 12$). Detailed data is presented in **Table 3**.

4. Discussion

Human lymphocytes and cell lines have been used as systems for the estimation of genotoxic damage by different agents or drugs [19]. Breast cancer is a common disease in mature women. In this study the average age of the patients was 47 years, which agrees with other authors such as Knaul *et al.* [2], who reported that 60% of women who die from this malignancy are between 30 and 59 years old, a heavy burden of early death.

Banerjee and Benedict [8] determined *in vitro* genotoxic damage using SCE in the A(T1) C1-3 hamster cell line exposed to antineoplastic agents such as Melphalan, Thiotepa, Actinomycin D, Bleomycin, Hycanthonone and 5-aza-C, obtaining a significant increase in SCE. Ocampo *et al.* [20] performed the assessment of *in vitro* genotoxic damage induced by Basuco in human lymphocytes, achieving a significant increase in the value of SCE in exposed cells. Regarding SCE, in this study was observed an increase of its value after chemotherapy, indicating a damage caused by the drugs to the DNA of patients as reported before [9] [18]. The fact that in this study was observed a higher frequency of SCE means that drugs are causing mutations that can lead to cell death at the level of lymphocytes, which is most likely the reflection of what is happening at the level of cancer cells.

In regard to MI and CPI, it is known that neoplastic cells have high values for these two biomarkers. This is due to the disarray or loss of control in cell proliferation [21] [22]. Based on this, it is expected that cells exposed to agents or drugs with a cytotoxic and cytostatic effect will show a decrease in RI, MI and CPI. Hick *et al.* [23], in a group of 20 argentinians exposed to arsenic, found a significant decrease in MI and CPI compared with a control group. The results of that study are similar to ours, as the statistical analysis resulted in a significant decrease in RI, MI and CPI obtained before versus after the first treatment dose. The decrease in the values of RI, MI and CPI means that the cycle of cells that have been exposed to antineoplastic drugs is lengthening. This is because if there is DNA damage under ordinary conditions, the cell cycle must stop at a check point to repair the damage, which in this case was caused by chemotherapy, therefore lengthening the cycle to promote the repair of damaged DNA [9].

The treatment assessed in this study is one of the main therapies used for breast cancer. Adriamycin and cyclophosphamide are intended to cause damage to the genetic material of neoplastic cells and thereby inhibit their

Table 3. Statistical analysis of SCE, RI, MI and CPI grouped according to treatment response.

Biomarker	Active Neoplasm (Median aQT) n = 4	Remission (Median aQT) n = 12	p Value
SCE	1.30	1.66	1.0
RI	1.155	1.085	0.504
MI	0.34	1.45	0.584
CPI	17.63	28.64	0.303

Mann-Whitney U test.

proliferation [12] [24]. *In vivo* studies for assessing the damage caused by treatment with certain drugs have been reported. Although these drugs had proven to be mutagenic, clastogenic and in general, genotoxic, *in vitro* tests are not currently performed in patients: personalized response is not evaluated because their effects depend on characteristics of each organism. In the present study, response to treatment was variable; most patients went into remission after completing the eight cycles of their treatment schedule. However, it was found that four patients had active neoplasms after two years of treatment, with CPI and MI behaving differently than in remission. That is, cytotoxicity and proliferation kinetics did not decrease after the first treatment, showing values above those found in patients in remission. These preliminary results invite to keep searching for biomarkers that may be indicative of early response to treatment.

In summary, treatment of breast cancer generally includes the application of 8 cycles of chemotherapy, adriamycin and cyclophosphamide being the most widely used scheme. Response to treatment is assessed after the third cycle, or at the end of it. The present study showed that there is an induction of SCE and a reduction in the frequency values of MI, RI and CPI after the first dose of adriamycin and cyclophosphamide. Therefore, these biomarkers have the possibility of being an early indicative of the treatment response. Moreover, this study showed that genotoxicity of adriamycin and cyclophosphamide can be induced and detected after the first cycle of treatment in breast cancer patients.

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Disclosure Statement

No competing financial interests exist.

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