Clinical Study

Serum Oxidative Stress Markers and Genotoxic Profile Induced by Chemotherapy in Patients with Breast Cancer: A Pilot Study

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The aim of this study was to evaluate the oxidative parameters of erythrocytes and genotoxicity in leukocytes of patients with breast cancer. Oxidative parameters were detected by spectrophotometry and genotoxic damage by single cell gel electrophoresis. Twenty-eight women with breast cancer were monitored before chemotherapy and after the second and fourth cycles of therapy with cyclophosphamide and doxorubicin. After the fourth cycle, increases \((P < 0.05)\) in the reactive substances to thiobarbituric acid levels, nitrite content, and superoxide dismutase activity and high rates of DNA damage in leukocytes were observed when compared with healthy women group and baseline levels. Similarly, after the second cycle, the same parameters were increased \((P < 0.05)\) when compared with baseline levels. Increase in catalase activity was detected only after the fourth cycle and reduced glutathione levels and glutathione peroxidase activity were decreased in all cycles when compared with healthy women, as well as after the second and fourth chemotherapy cycles compared to baseline \((P < 0.05)\). Patients with breast cancer presented an indicative of oxidative stress before, during, and after chemotherapy, as well as increased genotoxic damage in all stages of treatment, demonstrating the clinical applicability of this investigation.

1. Introduction

The etiology of breast cancer possesses a multifactorial origin [1, 2], showing as risk factors reproductive age, early menarche, late menopause, nulliparity, exogenous hormones, smoking, obesity, diet, alcohol consumption, physical inactivity, and genetic and environmental factors [1–5].

Most chemotherapeutic agents are not specific against neoplastic cells, also affecting normal cells [6], which results in a wide range of adverse reactions in virtually all tissues of body such as bone marrow suppression, alopecia, fatigue, generalized rash, diarrhea, and dizziness [7, 8]. Cyclophosphamide, one of the most used anticancer compounds, is a bifunctional alkylating member of the nitrogen mustard
family that induces various types of DNA damage, such as DNA adducts, gene mutations, and chromosomal aberrations [6, 9]. In clinical and trials protocols, cyclophosphamide is used in combination with doxorubicin, an anthracycline agent capable of intercalating into DNA [10]. Their mechanism of cytotoxicity includes intracellular production of free radicals, DNA intercalation, and subsequent inhibition of DNA topoisomerase II [6, 9, 10].

Reactive oxygen species (ROS) represent important factor in carcinogenesis and may play a role in initiation and progression of tumors. Free radicals stimulate oxidative DNA damage, contributing to mutagenesis, which is essential for the process of tumor initiation [11–13]. Unrepaired DNA damage has been associated with a variety of human disorders including cancer and neurodegenerative diseases. When DNA is properly repaired, the injuries are inactivated and the cells return to normal cell cycle operation. If this damage is not repaired, specific cellular responses such as cell death, senescence, or uncontrolled proliferation could result. This damage may consist of small lesions in very specific sites within the DNA molecule, as adducts, cross-links, abasic sites, and points of gross abnormalities [14, 15]. The extent of this damage caused by ROS can be maximized or minimized by enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) or nonenzymatic (vitamins A, C, and E, selenium, and reduced glutathione (GSH)) [2, 16–18] mechanisms of antioxidant defense. Based on this approach, the present study evaluated the antioxidant and genotoxic profile in blood cells of patients receiving a combined chemotherapy of adriamycin (doxorubicin) and cyclophosphamide (AC).

2. Materials and Methods

2.1. Study Population and Sample Collection. The subjects were patients diagnosed with ductal breast cancer under treatment at the Department of Oncology, São Marcos Hospital, Teresina, Piauí, Brazil, from August 2012 to February 2013. This clinical study was approved by the Research Ethics Committee of University Center UNINOVAFAPI (registration number 0406.0.043.00011). This study involved a total of 56 individuals including 28 patients exposed to chemotherapy by the AC protocol (adriamycin 60 mg/m² and cyclophosphamide 600 mg/m²) and 28 patients not exposed to the chemotherapy. The patients were exposed to four 21-day cycles with intravenous AC. The unexposed group consisted of individuals who had not been exposed to genotoxic agents (including radiation and chemicals) and who were free of any malignant neoplasm or clinical, biochemical, hematological, hepatic, cardiovascular, renal, or endocrine manifestations. Blood samples were collected with EDTA or heparin by venipuncture using vacutainers, maintained at 4°C during transport to the laboratory, and immediately processed. Three collections of the peripheral blood were carried out during four cycles of chemotherapy: the first collection was performed before the beginning of treatment (C0), 21 days after the second cycle of chemotherapy (C2), and 21 days after the fourth cycle (C4).

All individuals in this study were submitted to a questionnaire from International Commission for Protection against Environmental Mutagens and Carcinogens [19], which included questions regarding standard demographic data (e.g., age and gender), medical issues (e.g., exposure to X-rays, vaccinations, and medications), lifestyle (e.g., smoking, coffee and alcohol consumption, diet, etc.) and occupation, such as number of working hours per day and protective measures adopted (PPE). In all groups, individuals who smoked more than 20 cigarettes per day were considered smokers [20].

After the questionnaires, data were analyzed using SPSS 17.0. (Chicago: SPSS Inc.) and the demographic, medical and lifestyle were summarized in Table 1.

Details about clinical features, such as cancer site, clinical stage and HER-2/neu, ER (estrogen receptor), and PR (progesterone receptor) status, were obtained and analyzed from medical records. The descriptive statistics for such variables are listed in Table 2.

2.2. Comet Assay. The alkaline comet (single cell gel electrophoresis (SCGE)) assay was performed as described by Singh et al. [21] with modifications suggested by Tice et al. [22]. Blood cells (5 μL) were embedded in 95 μL of 0.75% low-melting point agarose, which was immediately added to the surface of a precoated (1.5% agarose) microscope slide. When the agarose had solidified, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris; pH 10.0–10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulfoxide (DMSO) for a minimum of 1 day and a maximum of 7 days. After treatment with lysis buffer, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA; pH N 13) for 20 min and the DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA after which the buffer was neutralized with 0.4 M Tris (pH 7.5) and dried overnight. Gels were rehydrated for 5 min in distilled water and then stained for 15 min (37°C) with a solution containing the following sequence: 34 mL of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, and 5% w/v sodium carbonate) and 66 mL of Solution A (5% sodium carbonate). The staining was stopped with 1% acetic acid and the gels were air dried. Analyses (100 cells/patient) were carried out by light microscopy (Olympus CX40) at 100× magnification with immersion oil. Images of cells (50 cells/slide in two replicates) were evaluated for the following: (i) damage index (DI), in which each cell was classified into classes (no damage = 0, maximum damage = 4) according to tail size and cell shape [23], with resulting values for each individual ranging from 0 (0 × 100) to 400 (4 × 100); (ii) damage frequency (DF), calculated as the percentage of injured cells. International guidelines and recommendations for the comet assay consider the visual scoring of comets to be a well-validated evaluation method. Although the DI parameter is often subjective, it has high correlation with computer-based image analysis [22, 24, 25].

2.3. Nitrite Content. The determination of nitrite content was based on the Griess reaction [26] in which 500 μL of Griess
Table 1: Demographic, medical, and lifestyle data of the patients.

| Characteristics                        | Control group | Breast cancer |
|----------------------------------------|---------------|---------------|
| Total of patients                      | 28 (100.0)    | 28 (100.0)    |
| Age in years (mean ± SD)               | 48.1 ± 11.8   | 50.8 ± 12.8   |
| Race [n (%)]                           |               |               |
| Caucasian                              | 20 (71.4)     | 22 (78.6)     |
| Non-Caucasian                          | 8 (28.6)      | 6 (21.4)      |
| Menopause [n (%)]                      |               |               |
| Premenopausal                          | 19 (67.9)     | 20 (71.4)     |
| Postmenopausal                         | 9 (32.1)      | 8 (28.6)      |
| Family history of breast cancer [n (%)]|               |               |
| Yes                                    | 6 (21.4)      | 15 (53.6)     |
| No                                     | 22 (78.6)     | 13 (46.4)     |
| Physical exercises [n (%)]             |               |               |
| Yes                                    | 11 (39.3)     | 9 (32.1)      |
| No                                     | 17 (60.7)     | 19 (67.9)     |
| Smoker [n (%)]                         |               |               |
| Never smoked                           | 20 (71.4)     | 12 (42.9)     |
| Ex-smoker                              | 8 (28.6)      | 13 (46.4)     |
| Smoking                                | 0 (0.0)       | 3 (10.7)      |
| Marital status [n (%)]                 |               |               |
| Single                                 | 7 (25.0)      | 6 (21.4)      |
| Married                                | 16 (57.1)     | 15 (53.6)     |
| Divorced                               | 1 (3.6)       | 3 (10.7)      |
| Widow                                  | 4 (14.3)      | 4 (14.3)      |

Table 2: Clinical characteristics of patients with breast ductal carcinoma (n = 28).

| Characteristics                        | Breast cancer |
|----------------------------------------|---------------|
| Cancer sites [n (%)]                   |               |
| Left breast                            | 11 (39.3)     |
| Right mama                             | 17 (60.7)     |
| Clinical stage [n (%)]                 |               |
| Grade 1                                | 4 (14.3)      |
| Grade 2                                | 10 (35.7)     |
| Grade 3                                | 14 (50.0)     |
| Estrogen receptor [n (%)]              |               |
| Negative                               | 7 (25.0)      |
| Positive                               | 21 (75.0)     |
| Progesterone receptor [n (%)]          |               |
| Negative                               | 7 (25.0)      |
| Positive                               | 21 (75.0)     |
| HER2/neu [n (%)]                       |               |
| Score 0                                | 9 (32.1)      |
| Score +1                               | 10 (35.7)     |
| Score +2                               | 1 (3.6)       |
| Score +3                               | 8 (28.3)      |

HER2/neu: human epidermal growth factor receptor 2.

reagent was added in white tube plus 500 μL of distilled water (Blank) was added in white tube. In another test tube 500 μL of Griess reagent and 500 μL of the homogenate at 10% of the erythrocytes (sodium phosphate buffer 50 mM pH 7.4) (Test) were added. The spectrophotometric measurement was performed at 560 nm. Results were expressed in μM/mg protein.

2.4. Thiobarbituric Acid Reactive Substances (TBARS) Levels. Blood samples were centrifuged at 3000 rpm at 4°C during 5 minutes. Plasma was removed and a pellet of erythrocytes was washed with a cold solution of NaCl 0.9% and centrifuged. An erythrocytes' homogenate 10% diluted in phosphate buffer sodium 50 mM and pH 7.4 was stored at −20°C. Lipid peroxidation was measured by TBARS levels, a method previously described by Draper and Hadley (1990) [27, 28]. 250 μL of homogenate, 1 mL of trichloroacetic acid 10%, and 1 mL of thiobarbituric acid 0.67% were mixed and stirred. Subsequently, this mixture was maintained in a bath of boiling water for 15 min and freshened under running water. After cooling, 2 mL of n-butanol was added and centrifuged at 1,200 rpm/5 min and the butanol phase was read spectrophotometrically at 535 nm. Results were expressed as nmol/mL.

2.5. Reduced Glutathione (GSH) Levels. Determination of GSH was based on the Ellman reaction (5,5′-dithiobis-2-nitrobenzoic acid) with some modifications described by Sedlak and Lindsay [29]. Four hundred microliters of erythrocytes' homogenate (EDTA pH 5.4 buffer) was mixed with
320 μL of distilled water and 80 μL of trichloroacetic acid 50%. After centrifugation at 3,000 rpm for 15 min, 400 μL from the supernatant was collected and added to 800 μL of Tris-HCl 0.4 M, pH 8.9, and 20 μL of DTNB 0.01 M. One minute later, spectrophotometric measurement was performed at 412 nm. Concentration of GSH was expressed in mg/g of hemoglobin.

2.6. Glutathione Peroxidase (GPx) Activity. The glutathione peroxidase activity coupled assay was determined by Baglia and Valentine [30]. GPx catalyzes the reduction of hydrogen peroxide (H₂O₂), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSGG). GSGG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP⁺ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPx is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPx concentration. 1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H₂O₂) of 1.0 μmole GSH to GSSG, per minute at 25°C, pH 7.0. Results were expressed in U/g of hemoglobin.

2.7. Catalase (CAT) Activity. Erythrocytes' homogenate in pH 7.4 was centrifuged (800 g, 20 min) and the supernatant was used to quantify catalase activity. The reaction medium was prepared with H₂O₂ (18 mL), Tris HCl 1 M, EDTA pH 8.0 5 mM (1.0 mL), and H₂O (0.8 mL). The reading was carried out in a quartz cuvette at 230 nm with 980 μL of reaction medium plus 20 μL erythrocytes' homogenate prepared in sodium phosphate buffer 50 mM, pH 7.4 [31].

2.8. Superoxide Dismutase (SOD) Activity. Erythrocytes homogenate prepared in sodium phosphate buffer 50 mM, pH 7.4, was centrifuged (800 g, 20 min) and supernatants were used for testing superoxide dismutase (SOD) activity. Cytochrome c reduction rate was determined by superoxide radicals using the xanthine-xanthine oxidase system as a source of superoxide anion (O₂⁻.) [32]. Results were expressed as U/mg protein. One unit (U) of SOD activity corresponds to the inhibition of 50% of O₂⁻ in the presence of cytochrome c.

2.9. Statistical Analyses. In order to determine statistical differences, data expressed as mean ± standard error of the mean (S.E.M.) were compared by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test (P < 0.05) using the Graphpad program (Intuitive Software for Science, San Diego, CA) and SPSS (version 19, SPSS Inc.). Correlations among data obtained were calculated using Spearman's correlation coefficient.

3. Results

3.1. Evaluation of Oxidative Stress. Evaluation of oxidative stress in patients with breast cancer in AC chemotherapy was performed by analyzing enzymatic and nonenzymatic parameters in erythrocytes by serum thiobarbituric acid reactive substances (TBARS) level, nitrite content, GSH concentration, and GPx, CAT, and SOD activities.

Results showed that the status of oxidative stress (P < 0.05) increased, as demonstrated by basal TBARS (1.42 ± 0.45 nM/mg of protein) and nitrite (1.16 ± 0.62 μM/mg protein) contents in erythrocytes of patients with breast cancer when compared with the control group (0.37 ± 0.09 nM/mg of protein and 0.16 ± 0.05 μM/mg of protein, resp.) (P < 0.05). When these same patients were submitted to chemotherapeutics (combination of cyclophosphamide and doxorubicin), such increases in both TBARS (4.76 ± 0.68 and 11.98 ± 0.65 nM/mg of protein) and nitrite ion levels (1.81 ± 0.02 and 3.49 ± 0.07 μM/mg of protein) were also detected in C2 and C4, respectively (P < 0.05; Table 3).

Red blood cells of the patients revealed decrease in reduced glutathione concentration at 36.1% (24.94 ± 1.51 U/g protein) in comparison with control group (36.13 ± 7.65 U/g protein) (P < 0.05). With AC chemotherapy, there was decrease in GSH levels in C2 (46.6%) and C4 groups (50.9%) (P < 0.05). Similarly, baseline levels (C0) also presented diminution of 21.4% and 27.7% in C2 and C4 groups, respectively (Table 3).

GPx activity (Figure 1(a)) showed reduction (63.32, 78.31, and 81.0%) in erythrocytes of the patients with breast cancer activity in all groups analyzed (101.90 ± 29.48, 60.25 ± 4.66, and 52.77 ± 3.26 U/g for C0, C2, and C4, resp.) when compared to the control group (277.8 ± 15.88 U/g), respectively (P < 0.05).

In relation to the catalase levels, only treated patients (C4, 22.83 ± 1.17 μM/mg) exhibited a significant increase (44.3%) when compared to the control group (15.82 ± 1.21 μM/mg), C0, and C2 (18.44 ± 1.24 and 18.77 ± 0.96 μM/mg, resp.) (Figure 1(b)). Similarly, superoxide dismutase activity (Figure 1(c)) also increased (33.1%) after the second cycle of chemotherapy (1.81 ± 0.63 μM/mg) when compared with control group. After chemotherapy (2.63 ± 0.65 μM/mg), its activity increased about 93.4 and 54.7% in relation to the control group (1.36 ± 0.62 μM/mg) and baseline (1.70 ± 0.43 μM/mg), respectively.

3.2. Index and Frequency of DNA Damage. DNA in the tail was organized into five classes: (i) class 0: undamaged, with no tail; (ii) class 1: with tail shorter than the diameter of the head (nucleus); (iii) class 2: with tail length between one and two times the diameter of the head; (iv) class 3: with tail longer than two times the diameter of the head; and (v) class 4: comets with no heads [33] (Figure 2).

With the application of alkaline comet assay it was possible to observe an increase (P < 0.05) in the classes of DNA damage in lymphocytes of patients with breast cancer (C0) in the control group. This condition is increased (P < 0.05) in C2 and C4 (Figure 3).

DNA damage in lymphocytes of patients with breast cancer increased by 122.6% (98.89 ± 5.56) compared to the control group (44.43 ± 1.67). After AC chemotherapy, there was an increase of 66.25 and 105.2% of damage index in C2 (18.77 ± 0.96 μM/mg, and doxorubicin), such increases in both TBARS (4.76 ± 0.68 and 11.98 ± 0.65 nM/mg of protein) and nitrite ion levels (1.81 ± 0.02 and 3.49 ± 0.07 μM/mg of protein) were also detected in C2 and C4, respectively (P < 0.05; Table 3).

An increase of 171.1% in frequency of DNA damage before chemotherapy (C0) (61.00 ± 2.01) was observed

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Table 3: Biomarkers levels of oxidative stress in antioxidant enzymatic system of patients with breast cancer before (C0), during (C2), and after chemotherapy (C4) and control group.

| Groups | TBARS levels (nM/mg de protein) | NO$_2^-$ content (µM/mg protein) | GSH concentration (U/g protein) |
|--------|---------------------------------|---------------------------------|-------------------------------|
| Control | 0.37 ± 0.09                     | 0.16 ± 0.05                     | 36.13 ± 7.65                  |
| C0     | 1.42 ± 0.45$^a$                | 1.16 ± 0.62$^a$                | 24.94 ± 1.51$^a$              |
| C2     | 4.76 ± 0.68$^{a,b}$            | 1.81 ± 0.02$^{a,b}$            | 19.30 ± 0.74$^{a,b}$          |
| C4     | 11.98 ± 0.65$^{a,b,c}$         | 3.49 ± 0.07$^{a,b,c}$          | 17.75 ± 0.46$^{a,b}$          |

TBARS: thiobarbituric acid reactive substances levels, NO$_2^-$: nitrite content, and GSH: reduced glutathione concentration. Values represent mean ± S.E.M. $^a P < 0.05$ when compared with control group (CG) by ANOVA followed by t-Student-Newman-Keuls. $^b P < 0.05$ when compared with C0 group (before chemotherapy) and $^c P < 0.05$ when compared with C2 group (second cycle of chemotherapy).

Figure 1: Antioxidant enzymes activity in erythrocytes of patients with breast cancer before (C0), during (C2), and after (C4) AC chemotherapy. Control group (CG) is represented by healthy patients. Values represent mean ± S.E.M. $^a P < 0.05$ when compared with control group (CG) by ANOVA followed by t-Student-Newman-Keuls. $^b P < 0.05$ when compared with C0 group (before chemotherapy) and $^c P < 0.05$ when compared with C2 group (second cycle of chemotherapy).

There was no correlation between sperm risk factors, age, smoking, and family history with the disease, as well as the levels of oxidative stress assessed by measurements of enzymes catalase, superoxide dismutase and glutathione peroxidase and malondialdehyde levels, nitrite, and reduced glutathione ($P > 0.05$). However, a significant positive
correlation (correlation factor = 0.389 and $P = 0.041$) was observed between race and nitrite levels after chemotherapy (Q4) and a negative correlation was observed with nitrite levels (correlation factor = $-0.474, P = 0.011$) in the diagnosis and activity of superoxide dismutase (correlation factor = $-0.389, P = 0.041$) after chemotherapy (Q4) to marital status. There was also a negative correlation (correlation factor = $-0.460, P = 0.014$) between the practice of physical exercises and malondialdehyde levels during chemotherapy. No correlation was observed between ER and PR receptors.
with oxidative stress, except between HER2 and glutathione peroxidase in Q2 group with 0.412 correlation factor $P = 0.29$.

Regarding the genotoxicity and oxidative stress, positive correlations were observed for the contents of DNA damage assessed at diagnosis (QD) compared to those obtained during (Q2) and after (Q4) chemotherapy, with 0.663 correlation factor 0.537 and $P = 0.000$ and 0.003, respectively. A negative correlation was observed between levels of DNA damage during chemotherapy (Q2) and nitrite levels (Q4), as well as between frequency of damage (QD) and nitrite in group Q4 and Q2 catalase group.

4. Discussion

Breast cancer is the second most common cancer in women over the age of 50. It is often first detected as an abnormality on a mammogram before the patient or health care provider feels it. Early cases may be asymptomatic, and pain and discomfort are typically not present. Breast cancer can begin in different areas of the breast, such as ducts and lobules. Ductal carcinoma in situ (DCIS) is the most common noninvasive or preinvasive type with chances of a recurrence under 30% within 5–10 years after initial diagnosis. On the other hand, invasive ductal carcinoma (IDC), known as infiltrating ductal carcinoma, is the most common type of invasive breast cancer, representing around 80% of cases. About two-thirds of women are 55 or older when they are diagnosed with an IDC [34].

Many studies have reported that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the etiology and progression of various cancers [35–38]. These reactive species have been associated with the development of carcinogenesis by activating diverse types of DNA damage, contributing to the emergence of mutations and chromosomal aberrations in the inflammatory process and leading to intense tissue disorganization and injuries [39].

An alternative method of analyzing oxidative stress is achieved by quantification of lipid peroxidation. The lipid radical is unstable and degrades very rapidly into secondary products. Most of them are electrophilic aldehydes, such as TBARS, which is the main marker of oxidative injury in the unsaturated lipids in cell membranes, leading to oxidation of fatty acids (LH) and formation of the lipid radical ($L\cdot$) [40]. Therefore, TBARS is an important indicator of oxidative stress [16]. The present study demonstrated an elevation of TBARS levels in AC-treated breast cancer patients compared to controls, corroborating previous studies [1, 35] and suggesting severe lipid peroxidation. These changes may be attributed to the production of hydroxyl radicals, which participate directly in the lipid peroxidation process, inducing a disturbance in membrane structure [41].

The evaluation of nitrite concentration has been used as an index of endogenous NO production in biological systems in distinct pathological processes beyond its physiological properties such as vasodilation, neurotransmission, and immune response [3, 18]. It was noted that serum nitrite content determined by the Griess method increased in patients with breast cancer. Higher levels of nitrite and nitrate are related to inflammation caused by diseases and pharmacotherapies [39]. Interestingly, the results of present study showed that, even before the chemotherapy cycles, the disease itself induced NO$_2^-$ generation and revealed an increase according to the treatment when compared to the baseline. Previous findings found analogous results, exhibiting increases in lipid oxidation activated by NO$_2^-$ levels or nitric oxide [5, 37]. Nitric oxide has a dual role in tumor invasion and metastasis, inducing tumor growth or promoting tumoricidal activity [3]. Our results support the
hypothesis that breast cancers are associated with increased nitric oxide levels whose changes are linked to inflammatory process [38]. Prior analyses in 14 patients with breast carcinomas showed no elevation of serum TBARS. However, increased NO concentrations were detected [42].

The extent of oxidative damage depends not only on ROS levels, but also on mechanisms of cellular antioxidant defenses. Low level of GSH, a molecule of critical importance in maintaining the stability of erythrocytes membranes, is related to cellular defense against xenobiotics and harmful compounds such as free radicals and hydroperoxides [43]. This drop in GSH was also observed in erythrocytes of the patients. An additional reduction in GSH levels was observed in healthy patients and those under chemotherapy. Glutathione acts as the first line of defense against free radicals produced by antitumor molecules. Decreased GSH levels can be explained by a decrease in GSH synthesis and/or increased consumption to remove peroxides and xenobiotics [44].

Metabolites generated by CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) induced lipid peroxidation by inactivation of GSH levels and SOD, CAT, GPX, and GST activities in erythrocytes of patients with breast cancer, thereby rendering the system inefficient in management of the free radical attack. Acrolein and phosphoramide mustard are the metabolites of cyclophosphamide that are among the causative agents, which reduce the activity of SOD, CAT, GPX, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase in erythrocytes of CMF treated breast cancer patients [45]. In the present study, GSH concentration and GPx activity were also observed just before AC chemotherapy. Our data demonstrate that GPx activity decreased, compared to the control group. However, this decrease was seen before the start of chemotherapy, suggesting no change in the activity of this enzyme for the therapeutic protocols used, since the reductions for during and after chemotherapy evaluation were similar to those observed prior to chemotherapy. Furthermore, these results suggest that the establishment of the pathophysiology of breast cancer may be a compromise in the activity of this enzyme. Present results and outcomes of Singh et al. [46] and Prabasheela et al. [47] also revealed, during chemotherapy FAC (5-fluorouracil, doxorubicin, and cyclophosphamide) or AC, a decrease in the nonenzymatic antioxidant GSH levels in patients with breast cancer before chemotherapy. On the other hand, additional studies did not find decrease in GPx activity before or after administration of chemotherapeutics [47, 48].

In relation to the CAT levels, our findings did not show differences before and during chemotherapy, presenting only increasing activity after treatment. On the other hand, while some studies found increases only after chemotherapy [48, 49], others observed decreases in CAT activity before and after chemotherapy [5, 40]. Since antioxidants can activate gene expression via the antioxidant response element [50], overexpression of enzymatic activities can explain these findings [40]. Similarly, SOD activity was elevated in patients with breast cancer before, during, and after chemotherapy. Hasan et al. [51] also showed plasma SOD activity increasing in patients with breast carcinoma compared to patients with benign tumors, suggesting that elevated total SOD might reflect a response to oxidative stress and then may predict a state of excess reactive oxygen species in the carcinogenesis process. Analogous outcomes were described by Badid et al. [52] before chemotherapy in erythrocytes of 38 patients with ductal breast cancer. Nevertheless, Gupta et al. [53] found a decrease in SOD activity in serum from 30 women. Patients with breast cancer in chemotherapy with epirubicin (90 mg/m²) and cyclophosphamide (600 mg/m²) also showed reduced CAT, SOD, GSH, and GPx activity and increased TBARS levels [54]. Some of these parameters are contradictory when compared to the outcomes in the present study. These differences are probably explained by the fact that enzymatic activity of antioxidant defenses is more expressed at the cytoplasmic and mitochondrial cellular level, especially for SOD [46].

In this study, the genotoxic profile assay of the patients with breast cancer under treatment with AC was also investigated. This evaluation was carried out by alkaline comet assay, a well-established, simple, versatile, rapid, visual, and sensitive tool used to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations [55]. Some other forms of DNA damage such as DNA cross-links (e.g., thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the comet assay. This technique has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing, and human biomonitoring [56]. Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assay, the advantages of the comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 10¹⁰ Da of DNA) [57].

The pathological condition significantly raised the damage indices and frequencies in lymphocytes when compared with the normal control group, confirming previous investigations performed by Sánchez-Suárez et al. [6] and Agnoletto et al. [4]. These effects on DNA structure remained elevated up to 80 days after the end of exposure to FEC (5-fluorouracil, epirubicin, and cyclophosphamide) [6]. As seen in this work, Vagh et al. [9] showed significant increase in DNA damage on lymphocytes of patients treated with cyclophosphamide.

In fact, antineoplastic agents are currently used in clinical studies which induce breaks in mammalian DNA strands as seen with topoisomerase I (camptothecin) and topoisomerase II (etoposide) inhibitors and 5-FU [58]. This, for example, is an antimetabolite widely used to treat breast adenocarcinoma and cancers of the gastrointestinal tract, head, and neck due to its inhibitory action on the enzyme thymidylate synthase, among other mechanisms, despite their in vivo clastogenic activity [59, 60]. Moreover, doxorubicin, beyond inhibiting topoisomerase II, also induces apoptosis and free radical formation [10, 45, 61]. These can cause DNA adducts, cross-links, double strand breaks, and single strand breaks. So, any biological reaction has potentiality to induce carcinogenesis [6, 62].
5. Conclusion

Patients with breast cancer under chemotherapy presented antioxidant status indicative of oxidative stress before, during, and after chemotherapy, as well as increasing genotoxic damage in all stages of the treatment. These results highlight the importance of monitoring patients in chemotherapy, especially using cytogenetic and molecular markers in order to provide new prognostic findings to the treatment as a strategy to reduce recurrences and to improve quality of life.

Ethical Approval

This study was previously approved by Committee in Ethical Research at UNINOVAFAPI (N. 0406.0.043.00011) and is in accordance with Brazilian research guidelines (Law 466/2012, National Council of Health, Brazil) and with Declaration of Helsinki.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] R. Kumaraguruparan, J. Kabaloomothy, and S. Nagini, “Correlation of tissue lipid peroxidation and antioxidants with clinical stage and menopausal status in patients with adenocarcinoma of the breast,” Clinical Biochemistry, vol. 38, no. 2, pp. 154–158, 2005.

[2] S. M. Tsai, M. F. Hou, S. H. Wu et al., “Expression of manganese superoxide dismutase in patients with breast cancer,” Kaohsiung Journal of Medical Sciences, vol. 27, pp. 167–172, 2011.

[3] A. Gönenç, D. Erten, S. Aslan, M. Akinçi, B. Şimşek, and M. Torun, “Lipid peroxidation and antioxidant status in blood and tissue of malignant breast tumor and benign breast disease,” Cell Biology International, vol. 30, no. 4, pp. 376–380, 2006.

[4] M. H. Agnoletto, T. N. Guecheva, F. Donđe et al., “Association of low repair efficiency with high hormone receptors expression and SOD activity in breast cancer patients,” Clinical Biochemistry, vol. 40, no. 16-17, pp. 1252–1258, 2007.

[5] K. A. Amin, B. M. Mohamed, M. A. M. El-Wakil, and S. O. Ibrahim, “Impact of breast cancer and combination chemotherapy on oxidative stress, hepatic and cardiac markers,” Journal of Breast Cancer, vol. 15, no. 3, pp. 306–312, 2012.

[6] P. Sánchez-Suárez, P. Ostrosky-Wegman, F. Gallegos-Hernández et al., “DNA damage in peripheral blood lymphocytes in patients during combined chemotherapy for breast cancer,” Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis, vol. 640, no. 1-2, pp. 8–15, 2008.

[7] E. P. M. de Almeida, M. G. R. de Gutiérrez, and N. P. Adami, “Monitoramento e avaliação dos efeitos colaterais da quimioterapia em pacientes com câncer de cólon,” Revista Latino-Americana de Enfermagem, vol. 12, no. 5, pp. 760–766, 2004.

[8] C. M. Walko and C. Grande, “Management of common adverse events in patients treated with Sorafenib: nurse and pharmacist perspective,” Seminars in Oncology, vol. 41, supplement 2, pp. S17–S28, 2014.

[9] H. Vaghef, P. Nygren, C. Edling, J. Bergh, and B. Hellman, “Alkaline single-cell gel electrophoresis and human biomonitoring for genotoxicity: a pilot study on breast cancer patients undergoing chemotherapy including cyclophosphamide,” Mutation Research—Genetic Toxicology and Environmental Mutagenesis, vol. 395, no. 2-3, pp. 127–138, 1997.

[10] S. Marsh and G. Liu, “Pharmacokinetics and pharmacogenomics in breast cancer chemotherapy,” Advanced Drug Delivery Reviews, vol. 61, pp. 381–387, 2009.

[11] J. Sastre-Serra, A. Valle, M. M. Company, I. Garau, J. Oliver, and P. Rocca, “Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer,” Free Radical Biology and Medicine, vol. 48, no. 4, pp. 506–512, 2010.

[12] L. Vera-Ramirez, P. Sanchez-Rovira, M. C. Ramirez-Tortosa et al., “Does chemotherapy-induced oxidative stress improve the survival rates of breast cancer patients?” Antioxidants and Redox Signaling, vol. 15, no. 4, pp. 903–909, 2011.

[13] L. R. Freeman and J. N. Keller, “Oxidative stress and cerebral endothelial cells: regulation of the blood-brain-barrier and antioxidant based interventions,” Biochimica et Biophysica Acta—Molecular Basis of Disease, vol. 1822, no. 5, pp. 822–829, 2012.

[14] H. Rodríguez-Rocha, A. García-García, M. I. Panayiotidis, and R. Franco, “DNA damage and autophagy,” Mutation Research, vol. 711, no. 1-2, pp. 158–166, 2011.

[15] J. Vijg, “Somatic mutations and aging: a re-evaluation,” Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, vol. 447, no. 1, pp. 117–135, 2000.

[16] N. Badjatia, A. Satyam, P. Singh, A. Seth, and A. Sharma, “Altered antioxidant status and lipid peroxidation in Indian patients with urothelial bladder carcinoma,” Urologic Oncology, vol. 28, no. 4, pp. 360–367, 2010.

[17] C. Glorieux, N. Dejeans, B. Sid, R. Beck, P. B. Calderon, and J. Verrax, “Catalase overexpression in mammary cancer cells leads to a less aggressive phenotype and an altered response to chemotherapy,” Biochemical Pharmacology, vol. 82, no. 10, pp. 1384–1390, 2011.

[18] A. Gönenç, A. Hacişevki, S. Aslan, M. Torun, and B. Şimşek, “Increased oxidative DNA damage and impaired antioxidant defense system in patients with gastrointestinal cancer,” European Journal of Internal Medicine, vol. 23, no. 4, pp. 350–354, 2012.

[19] A. V. Carrano and A. T. Natarajan, “Considerations for population monitoring using cytogenetic techniques International Comission for protection against Environmental Mutagens and Carcinogens (ICPEMC publication 14),” Mutation Research, vol. 204, no. 3, pp. 379–406, 1988.

[20] H. Hoffmann, J. Högel, and G. Speit, “The effect of smoking on DNA effects in the comet assay; a meta-analysis,” Mutagenesis, vol. 20, no. 6, pp. 455–466, 2005.

[21] N. P. Singh, M. T. McCoy, R. R. Tice, and E. L. Schneider, “A simple technique for quantitation of low levels of DNA damage in individual cells,” Experimental Cell Research, vol. 175, no. 1, pp. 184–191, 1988.
[22] R. R. Tice, E. Agurell, D. Anderson et al., "Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing," *Environmental and Molecular Mutagenesis*, vol. 35, no. 3, pp. 206–221, 2000.

[23] O. Garcia, T. Mandina, A. I. Lamadrí et al., "Sensitivity and variability of visual scoring in the comet assay: results of an inter-laboratory scoring exercise with the use of silver staining," *Mutation Research*, vol. 556, no. 1-2, pp. 25–34, 2004.

[24] A. R. Collins, "The comet assay for DNA damage and repair: principles, applications, and limitations," *Applied Biochemistry and Biotechnology*, Part B Molecular Biotechnology, vol. 26, no. 3, pp. 249–261, 2004.

[25] M. Dusinska and A. R. Collins, "The comet assay in human biomonitoring: gene-environment interactions," *Mutagenesis*, vol. 23, no. 3, pp. 191–205, 2008.

[26] L. C. Green, S. R. Tannenbaum, and P. Goldman, "Nitrate synthesis in the germfree and conventional rat," *Science*, vol. 212, no. 4490, pp. 56–58, 1981.

[27] E. D. Wills, "Mechanisms of lipid peroxide formation in animal tissues," *Biochemical Journal*, vol. 99, no. 3, pp. 667–676, 1966.

[28] H. H. Draper and M. Hadley, "Malondialdehyde determination as an index of lipid peroxidation," in *Methods in Enzymology*, vol. 186, pp. 421–431, 1990.

[29] J. Sedlak and R. H. Lindsay, "Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent," *Analytical Biochemistry*, vol. 25, no. 1, pp. 192–205, 1968.

[30] D. E. Paglia and W. N. Valentine, "Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase," *The Journal of Laboratory and Clinical Medicine*, vol. 70, no. 1, pp. 158–169, 1967.

[31] B. Chance and A. C. Maehly, "Assay catalases and peroxidases," *Methods in Enzymology*, vol. 2, pp. 764–768, 1995.

[32] J. R. Arthur and R. Boyne, "Superoxide dismutase and glutathione peroxidase activities in neutrophils from selenium deficient and copper deficient cattle," *Life Sciences*, vol. 36, no. 16, pp. 1569–1575, 1985.

[33] S. T. Miorelli, R. M. Rosa, D. J. Moura et al., "Antioxidant and anti-mutagenic effects of esbenol in yeast and in cultured mammalian V79 cells," *Mutagenesis*, vol. 23, no. 2, pp. 93–99, 2008.

[34] E. A. Rakha, J. S. Reis-Filho, F. Baehtner et al., "Breast cancer prognostic classification in the molecular era: the role of histological grade," *Breast Cancer Research*, vol. 12, pp. 1–12, 2010.

[35] D. Pande, R. Negi, S. Khanna, R. Khanna, and H. D. Khanna, "Vascular endothelial growth factor levels in relation to oxidative damage and antioxidant status in patients with breast cancer," *Journal of Breast Cancer*, vol. 14, no. 3, pp. 181–184, 2011.

[36] S. J. Ralph, S. Rodríguez-Enríquez, J. Neuzil, E. Saavedra, and R. Moreno-Sánchez, "The causes of cancer revisited: ‘Mitochondrial malignancy’ and ROS-induced oncogenic transformation—why mitochondria are targets for cancer therapy," *Molecular Aspects of Medicine*, vol. 31, no. 2, pp. 145–170, 2010.

[37] G. Rockenbach, P. F. di Pietro, C. Ambrosi et al., "Dietary intake and oxidative stress in breast cancer: before and after treatments," *Nutrition Hospitalaria*, vol. 26, no. 4, pp. 737–744, 2011.

[38] C. G. Vallejo, A. Cruz-Bermúdez, P. Clemente, R. Hernández-Sierra, R. Garesse, and M. Quintanilla, "Evaluation of mitochondrial function and metabolic reprogramming during tumor progression in a cell model of skin carcinogenesis," *Biochimie*, vol. 95, no. 6, pp. 1171–1176, 2013.

[39] N. Gupta, B. Goswami, and P. Mittal, "Effect of standard anthracycline based neoadjuvant chemotherapy on circulating levels of serum IL-6 in patients of locally advanced carcinoma breast—a prospective study," *International Journal of Surgery*, vol. 10, no. 10, pp. 638–640, 2012.

[40] T. T. Reed, "Lipid peroxidation and neurodegenerative disease," *Free Radical Biology and Medicine*, vol. 51, no. 7, pp. 1302–1319, 2011.

[41] C. Panis, A. C. S. A. Herrera, V. J. Victorino et al., "Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy," *Breast Cancer Research and Treatment*, vol. 133, no. 1, pp. 89–97, 2012.

[42] H. Alagöl, E. Erdem, B. Sancak, G. Türkmcn, M. Camlibel, and G. Bugdayci, "Nitric oxide biosynthesis and malondialdehyde levels in advanced breast cancer," *Australian and New Zealand Journal of Surgery*, vol. 69, no. 9, pp. 647–650, 1999.

[43] A. Pastore, G. Federici, E. Bertini, and F. Piemonte, "Analysis of glutathione: implication in redox and detoxification," *Clinica Chimica Acta*, vol. 333, no. 1, pp. 19–39, 2003.

[44] S. Aggarwal, M. Subberwal, S. Kumar, and M. Sharma, "Brain tumor and role of β-carotene, α-tocopherol, superoxide dismutase and glutathione peroxidase," *Journal of Cancer Research and Therapeutics*, vol. 2, no. 1, pp. 24–27, 2006.

[45] S. Subramaniam, S. Subramaniam, and C. S. Shyamala Devi, "Erythrocyte antioxidant enzyme activity in CMF treated breast cancer patients," *Breast Cancer Biophysics*, vol. 14, no. 3, pp. 177–182, 1994.

[46] G. Singh, S. K. Maulik, A. Jaiswal, P. Kumar, and R. Parshad, "Effect on antioxidant levels in patients of breast carcinoma during neoadjuvant chemotherapy and mastectomy," *Malaysian Journal of Medical Sciences*, vol. 17, no. 2, pp. 24–28, 2010.

[47] A. B. Prabasheela, A. K. Singh, A. Fathima, K. Pragulbh, N. J. Deka, and R. Kumar, "Association between antioxidant enzymes and breast cancer," *Recent Research in Science and Technology*, vol. 3, no. 11, pp. 93–95, 2011.

[48] J. Kasapović, S. Pejić, V. Stojiljković et al., "Antioxidant status and lipid peroxidation in the blood of breast cancer patients of different ages after chemotherapy with 5-fluorouracil, doxorubicin and cyclophosphamide," *Clinical Biochemistry*, vol. 43, no. 16-17, pp. 1287–1293, 2010.

[49] C. P. Rajnesh, A. Manimaran, K. R. Sasikala, and P. Adaikappen, "Lipid peroxidation and antioxidant status in patients with breast cancer," *Singapore Medical Journal*, vol. 49, no. 8, pp. 640–643, 2008.

[50] M. Gago-Dominguez, J. E. Castelao, M. C. Pike, A. Sevanian, and R. W. Haile, "Role of lipid peroxidation in the epidemiology and prevention of breast cancer," *Cancer Epidemiology Biomarkers and Prevention*, vol. 14, no. 12, pp. 2829–2839, 2005.

[51] H. R. Hasan, T. H. Mathkor, and M. H. Al-Habal, "Superoxide dismutase isozyme activities in plasma and tissues of Iraqi patients with breast cancer," *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 6, pp. 2571–2576, 2012.

[52] N. Badid, F. Z. Baba Ahmed, H. Merzouk et al., "Oxidant/antioxidant status, lipids and hormonal profile in overweight women with breast cancer," *Pathology and Oncology Research*, vol. 16, no. 2, pp. 159–167, 2010.

[53] R. K. Gupta, A. K. Patel, R. Kumari et al., "Interactions between oxidative stress, lipid profile and antioxidants in breast cancer: a Case Control Study," *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 12, pp. 6295–6298, 2012.
[54] M. J. Ramírez-Expósito, E. Sánchez-López, C. Cueto-Ureña et al., "Circulating oxidative stress parameters in pre- and post-menopausal healthy women and in women suffering from breast cancer treated or not with neoadjuvant chemotherapy," *Experimental Gerontology*, vol. 58, pp. 34–42, 2014.

[55] P. L. Olive and J. P. Banáth, "The comet assay: a method to measure DNA damage in individual cells," *Nature Protocols*, vol. 1, no. 1, pp. 23–29, 2006.

[56] P. Møller, "The alkaline Comet assay: towards validation in biomonitoring of DNA damaging exposures," *Basic & Clinical Pharmacology & Toxicology*, vol. 98, no. 4, pp. 336–345, 2006.

[57] C. M. Gedik, S. W. B. Ewen, and A. R. Collins, "Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells," *International Journal of Radiation Biology*, vol. 62, no. 3, pp. 313–320, 1992.

[58] A. N. C. Sortibrán, M. G. O. Téllez, and R. R. Rodríguez-Arnaiz, "Genotoxic profile of inhibitors of topoisomerases I (camptothecin) and II (etoposide) in a mitotic recombination and sex-chromosome loss somatic eye assay of *Drosophila melanogaster*," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 604, no. 1-2, pp. 83–90, 2006.

[59] G. M. Zúñiga-González, O. Torres-Bugarín, A. L. Zamora-Perez et al., "Induction of micronucleated erythrocytes in mouse peripheral blood after cutaneous application of 5-fluorouracil," *Archives of Medical Research*, vol. 34, no. 2, pp. 141–144, 2003.

[60] P. Noordhuis, U. Holwerda, C. L. van der Wilt et al., "5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers," *Annals of Oncology*, vol. 15, no. 7, pp. 1025–1032, 2004.

[61] L. P. Swift, A. Rephaeli, A. Nudelman, D. R. Phillips, and S. M. Cutts, "Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death," *Cancer Research*, vol. 66, no. 9, pp. 4863–4871, 2006.

[62] N. Suhail, N. Bilal, H. Y. Khan et al., "Effect of vitamins C and E on antioxidant status of breast-cancer patients undergoing chemotherapy," *Journal of Clinical Pharmacy and Therapeutics*, vol. 37, no. 1, pp. 22–26, 2012.