Oxidative stress parameters in women with breast cancer undergoing neoadjuvant chemotherapy and treated with nutraceutical doses of oral glutamine[1]

Parâmetros de estresse oxidativo em mulheres com câncer de mama submetidas à quimioterapia neoadjuvante e tratadas com doses nutracêuticas da glutamina oral[1]

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ABSTRACT
PURPOSE: To evaluate the effects of oral administration of GLN on the oxidative stress in women with breast cancer undergoing neoadjuvant FAC chemotherapy (5 fluouracil 500 mg/m^2+Doxorubicin 50 mg/m^2+Cyclophosphamide 500 mg/m^2 body surface area).
METHODS: Twenty women (mean age: 51.7 years) with breast ductal carcinomas classified as T3 or T4 were included in the study, regardless of pre or post menopause status. Sachets containing glutamine 15g ("A") or milk protein 15g ("B") were prepared by a registered pharmacist. Allocation of patients was made by software program. Patients who received sachets labeled "A" were included in G1 group. The remaining patients, treated with the preparation labeled "B", were included in group G2. Sachets contents were blended in 150 ml of drinking water, and were given daily to each patient during the entire course of neoadjuvant chemotherapy. Peripheral blood samples were collected in the first day of each of the three cycles of chemotherapy before drug infusion. Tumor and normal breast samples were collected at the end of Patey´s surgical procedure. Samples were analysed for GSH and TBARS contents.
RESULTS: TBARS and GSH values were not different in breast healthy and tumor tissues nor blood when comparing control (G-2) and glutamine-treated (G-1) patients. Also, no significant differences were found in TBARS and GSH levels comparing different timepoints within the same group.
CONCLUSION: Oral GLN (15g/kg/day) offers no protection against systemic or local oxidative stress in women with breast Ca undergoing neoadjuvant chemotherapy (FAC).
Keywords: Breast Neoplasms. Women. Drug Therapy. Oxidative Stress. Glutamine.

RESUMO
OBJETIVO: Avaliar os efeitos da administração oral de GLN sobre o estresse oxidativo em mulheres com câncer mamário submetidas à quimioterapia neoadjuvante com esquema FAC (5 fluouracil 500 mg/m2+doxorrubicina 50 mg/m2+ciclofosfamida 500 mg/m2 de superfície corporal).
MÉTODOS: Vinte mulheres (idade média: 51,7 anos) com carcinoma ductal de mama, classificado como T3 ou T4 foram incluídas no estudo, independente do seu estado menstrual. Embalagens contendo 15g de glutamina ou proteína do leite foram preparadas por farmacêutico. Alocação dos pacientes foi feita na seqüência gerada por "software". Pacientes que receberam embalagens tipo "A" foram incluídas no grupo G1. Pacientes tratadas com a preparação denominada "B", foram incluídas no grupo G2. O material foi misturado com uso de liquidificador em 150 ml de água potável, e administrado diariamente aos pacientes durante todo o curso da quimioterapia neoadjuvante (esquema FAC). Amostras de sangue periférico foram coletadas no inicio dos três ciclos de quimioterapia, antes da infusão de drogas. Amostras de tumor e tecido mamário normal foram colhidas no final do procedimento cirúrgico (cirurgia de Patey). As amostras foram analisadas para determinação das concentrações de GSH e TBARS.
RESULTADOS: Concentrações de TBARS/ GSH não foram diferentes no tumor. tecido mamário ou sangue, comparando os grupos G-2 vs.G-1. Além disso, não foram encontradas diferenças significativas nos níveis de TBARS e GSH comparando momentos diferentes dentro do mesmo grupo.
CONCLUSÃO: GLN (15g/kg/dia) administrada por via oral não oferece proteção contra o estresse oxidativo sistêmico ou local em mulheres com câncer de mama, submetidas à quimioterapia neoadjuvante (FAC).
Descritores: Neoplasias da Mama. Mulheres. Quimioterapia. Estresse Oxidativo. Glutamina.

Introduction

Breast cancer is the second most common cancer worldwide and more common among women, accounting for 22% of new cases in this group. In Brazil, 49,400 new cases are expected in 2010, with an estimated risk of 49 cases per 100 000 women.[1]

Oxidative stress affects cell structure and function by inducing lipid, carbohydrate, protein and DNA damage in biological systems.[2] Reactive oxygen substances (ROS) are chemically reactive molecules that are produced endogenously during various cellular metabolic activities. [3] These free radicals tend to be eliminated from the body by all the enzymes glutathione peroxidase, glutathione reductase, superoxide dismutase and by catalase. Thus, under physiological conditions, the balance between pro-oxidant agents and antioxidant defenses is attained. [4] When the balance cannot be established, oxidative stress occurs. To the living organisms remain three alternatives: a) adaptation, due to increased activity of antioxidant systems; b) tissue injury, lipids, carbohydrates or proteins damage, c) cell death by necrosis or apoptosis.[5] Among well documented effects, free radicals are known to induce DNA damage and genomic instability favoring the acquisition of mutations that contribute to cellular transformation and cancer cells survival.[6-8] ROS modifies redox-sensitive signal transduction pathways that contribute in the cell transformation, cell growth and cancer progression. [9]

Antioxidants are substances capable of inhibiting oxidation. The antioxidant mechanism involves substances that prevent the generation of reactive oxygen species or capture them to prevent their interaction with cellular targets. This group of substances is represented by antioxidant enzymes, proteins and chelating agents such as transferrin and ceruloplasmin and nonenzymatic substances such as glutathione (GSH).[10] Glutamine (GLN) is a conditionally essential nutrient during sepsis or trauma[11] and the most abundant amino acid in plasma and skeletal muscle.[12]

Previous studies have demonstrated that permanent modification of genetic material resulting from the oxidative damage leads to carcinogenesis.[13-15] Studies have demonstrated that GLN decreases intracellular levels of GSH in tumor cells, making tumor cells more sensitive to radiation and chemotherapy while restoring the depressed levels of GSH in normal host tissues, thereby improving the overall host well-being and resulting in decreased morbidity and mortality associated with cancer and its treatment.[16] Unlike other amino acids, the concentration of Gln in plasma can approach 1 mM, and this abundance allows it to serve as a major shuttle for the inter-organ traffic of both carbon and nitrogen. Recent data[17] have demonstrated that Gln metabolism buffers cells against bioenergetic and oxidative stress, mediates inter- and intracellular signaling, and promotes quality control in macromolecules and organelles. These GLN roles justify this study.

Methods

Patients

This prospective, randomized, controlled, double-blind study, clinically staged by UICC-TNM system[18] was approved by the local Ethics Committee – COMEPE, protocol #01/2005, February 10, 2005 and conducted in compliance with the Helsinki Declaration of 1975, as revised in 2008 (World Medical Association www.wma.net/e/policy/b3.htm) and Resolution 196/96 of the Brazilian National Health Service (http://conselho.saude.gov. br/resolucoes/reso_96.htm).

Written informed consent was obtained from all patients. Twenty women (mean age: 51.7 years, age range 32.1-75.4 years at the time of surgery, with breast ductal carcinomas classified as T3 (tumor more than 5 cm in greatest dimension) or T4 (tumor of any size with direct extension to chest wall or skin), were included in the study, regardless of pre or post menopause status. Patients who had distant metastatic disease (M1) at time of diagnosis, who have asked to leave and / or fail to regularly follow the steps planned for the study as well as patients with disease progression at any stage of the treatment were excluded from the study.

Study groups

In order to blind all people involved in the study, identical aluminum foil sachets labeled "A" and "B", containing glutamine 15g or milk protein 15g were prepared by a registered pharmacist. The contents of each sachet was recorded and stored for future identification. Allocation of patients was made by software program (www.lee.dante.br). Patients who received sachets labeled "A" were included in G1 group. The remaining patients, treated with the preparation labeled "B", were included in group G2. Only the responsible pharmacist was aware of the contents of "A" and "B" sachets. The mixture, dissolved in 150 ml of drinking water, was given daily to each patient during the entire course of chemotherapy. All patients underwent three cycles of neoadjuvant chemotherapy with FAC regimen (5 fluouracil 500 mg/m^2 + Doxorubicin 50 mg/m^2 + Cyclophosphamide 500 mg/m^2 body surface area), administered as a *bolus* each 21 days. After completion of pre-operative chemotherapy, the patients were subjected to the standard surgical treatment for locally advanced female breast cancer (Patey procedure[19]), which consists of a total mastectomy with axillary linfonode excision, levels I, II and III. Peripheral blood samples were collected in the first day of each of the three cycles of chemotherapy before drug infusion (T1, T2, T3). Tumor tissue and normal breast tissue were collected at the end of surgical procedure. Tissue and blood samples were stored at -70° for GSH and thiobarbituric acid reactive substances (TBARS) assays. After surgery, the patients received postoperative adjuvant radiotherapy, which included irradiation of residual breast, internal mammary lymph node chain and supraclavicular fossa. Following completion of radiotherapy, three more cycles of adjuvant chemotherapy were performed, at the same dosages of neoadjuvant chemotherapy.

Chemicals and drugs

L-Glutamine (L-Gln) was purchased from Ajinomoto/ Brazil. All other chemicals were purchased from standard commercial sources and were of the highest quality available.

Biochemical analysis

TBARS[20] and GSH[21] concentrations were measured according to biochemical methods published elsewhere.

Statistical analysis

Comparisons between control (G-2) and glutamine groups (G1) were made using the Mann-Whitney *U* test). ANOVA (Friedman test) followed by Dunn's Multiple Comparison Test was used compare all timepoints**.** GSH and TBARS tissue samples contents were compared with Mann-Whitney *U* test. In all cases, the level of significance was set at 5%. $P<0.05$ was considered statistically significant.

Results

Eleven patients (55%) had left breast tumor and in 9 patients (45%) the right breast was affected. Tumors were classified as T2 in 5 patients (25%), T3 in 4 patients (20%), T4a in 1 patient (5%), T4b in 8 patients (40%) and T4d in 2 patients (10%). Regarding the hormonal *status*, 10 patients (50%) were pre-menopausal, 5 patients (25%) were menopausal and 5 patients (25%) were postmenopausal women.

TBARS and GSH values were not different in breast healthy and tumor tissues when comparing control (G-2) and glutamine-treated (G-1) patients (Figures 1-2).

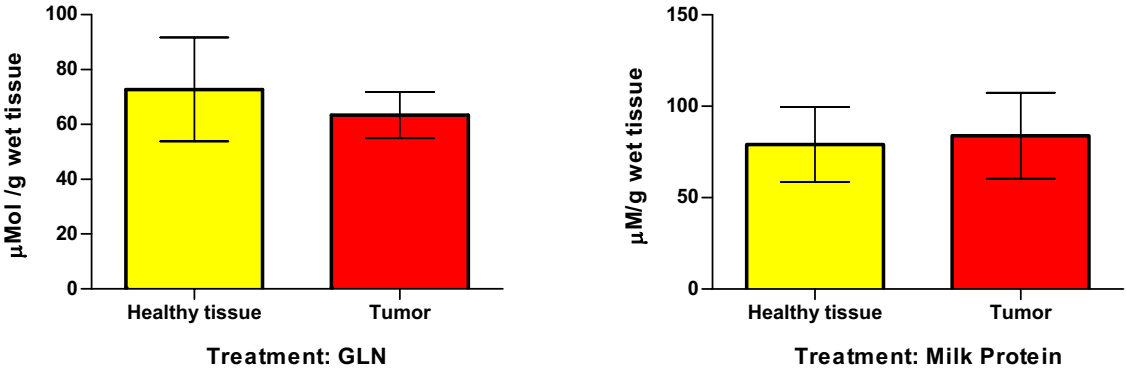

FIGURE 1 - Reduced glutathione (GSH) concentrations (micromoles of GSH per gram of wet tissue) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (healthy tissue, yellow bars) and tumor tissue (red bars). GSH levels were not different by Mann Whitney test.

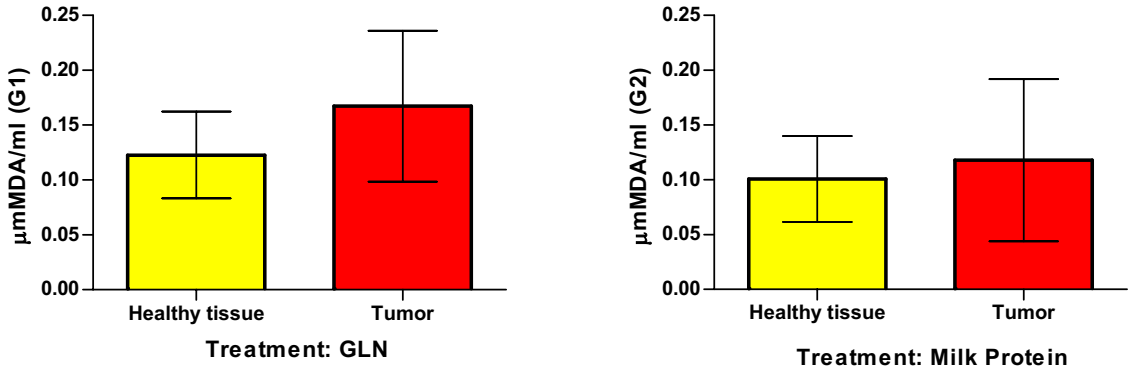

FIGURE 2 - Thiobarbituric acid-reactive substances concentrations (micromoles of malondialdehyde per per gram of wet tissue) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (healthy tissue, yellow bars) and tumor tissue (red bars). TBARS levels were not different by Mann Whitney test.

No statistically significant differences were found in blood TBARS and GSH levels comparing control (G-2) and glutamine-treated (G-1) patients (Figures 3 and 5). Also no significant differences were found in TBARS and GSH levels comparing different timepoints within the same group (Figures 4 and 6).

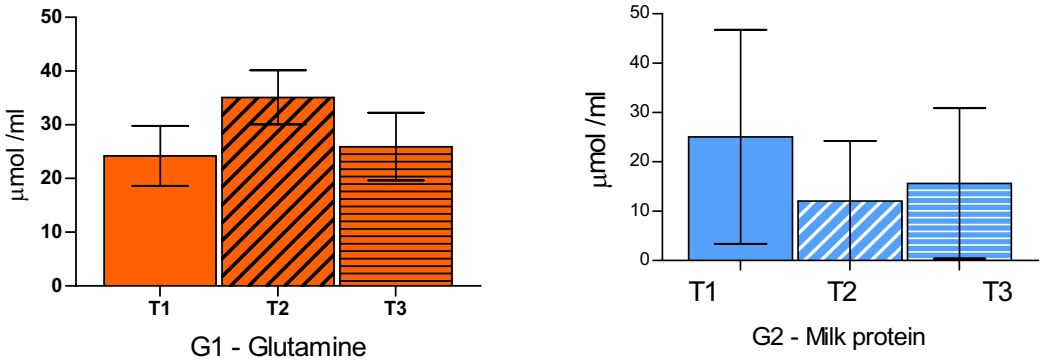

FIGURE 3 - Reduced glutathione (GSH) concentrations (micromoles of GSH per ml of plasma) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (blue bars) and GLN (orange bars) groups in T1, T2 and T3 time-points. GSH levels were not different within groups by ANOVA test (Friedman/Dunn test).

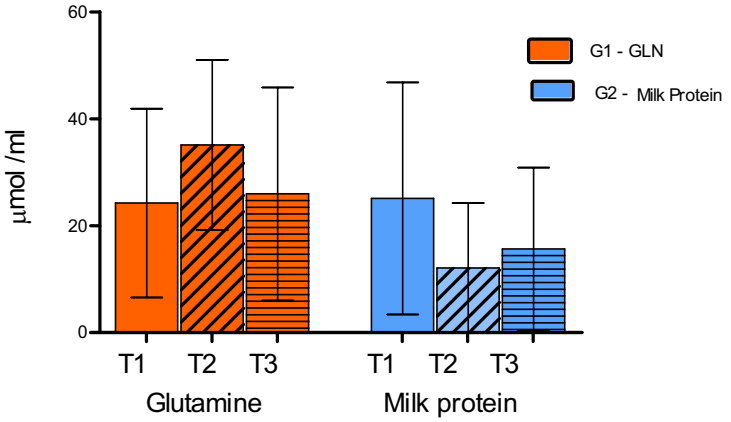

FIGURE 4 - Reduced glutathione (GSH) concentrations (micromoles of GSH per ml of plasma) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (blue bars) and GLN (orange bars) groups in T1, T2 and T3 time-points. GSH levels were not different comparing G1 versus G2 groups by ANOVA test (Friedman/Dunn test).

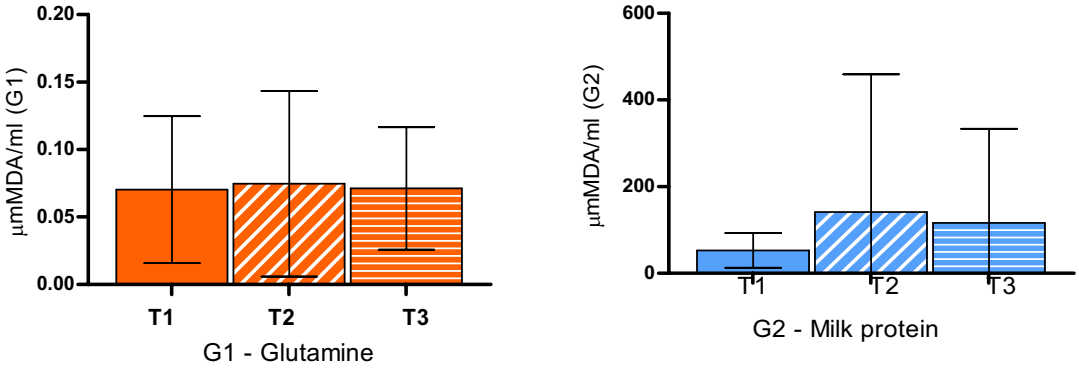

FIGURE 5 - Thiobarbituric acid-reactive substances concentrations (micromoles of malondialdehyde per ml of plasma) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (blue bars) and GLN (orange bars) groups in T1, T2 and T3 time-points. TBARS levels were not different within groups by ANOVA test (Friedman/Dunn test).

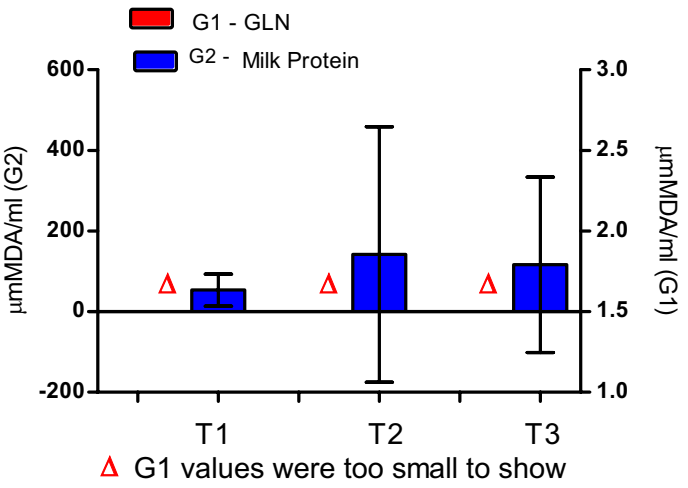

FIGURE 6 - Thiobarbituric acid-reactive substances concentrations (micromoles of malondialdehyde per ml of plasma) in GLN (G1) and milk protein (G2) treated patients. Bars represent mean ± SD of control (blue bars) and GLN (orange bars) groups in T1, T2 and T3 time-points. TBARS levels were not different comparing G1 versus G2 groups by ANOVA test (Friedman/Dunn test).

Discussion

Breast cancer is the leading cause of cancer death among women in the industrialized countries.[22] Several lines of evidence strongly suggest the involvement of cellular oxidative stress in carcinogenic processes.[23-24] Studies carried out both in human and experimental animals show that lipid peroxidation (LP) has a very important role in the initiation and promotion of cancer. The damage to the defensive systems has been shown to induce LP in the course of carcinogenesis.[25] Increased LP products in breast cancer tissues have been demonstrated.[26]

Tumor cells have been shown to have abnormal levels of antioxidant enzyme activities when compared with normal cells.[27] Enzyme activities were not evaluated in our study. However, the levels of GSH and TBARS in samples removed from healthy breast tissue and tumor were not different (Figures 1-2). Published studies implicate free radicals in cell transformation and in the uncontrolled growth potential of tumor cells.[6, 26,28] Among well documented effects, free radicals are known to induce DNA damage and genomic instability favoring the acquisition of mutations that contribute to cellular transformation and cancer cells survival.[6-7,26] ROS modifies redox-sensitive signal transduction pathways that contribute in the cell transformation, cell growth and cancer progression.[9] Permanent modification of genetic material resulting from the oxidative damage is one of the vital steps involved in mutagenesis that leads to carcinogenesis. Stimulation of DNA damage can either arrest or induce transcription, signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis.[6, 29-30]

Protection provided by GLN against oxidative damage follows two known pathways. First, Gln donates both carbon and nitrogen to glutathione (GSH), the major intracellular antioxidant. Second, Gln metabolism provides a source of reducing equivalents (NADPH) necessary to maintain GSH in its reduced state.[17] Studies have demonstrated that breast cancers show high levels of oxidative stress as verified by the detection of oxidative DNA adducts in breast cancer tissue[31] or a significant raise in oxidative stress markers in the plasma from breast cancer patients.[32] Moreover, the use of chemotherapy increases oxidative stress[33] FAC chemotherapy and radiotherapy promote further oxidative shift, which potentiate already existing chronic oxidative stress linked to breast cancer.[33]

Whereas the protective action of GLN in cancer bearing experimental animals has been demonstrated,[16] one would expect a similar effect in humans. It is questioned why GSH levels did not increase in this study. The answer may be related to dose of GLN used. In this study, patients in the glutamine group received daily doses of 15g of GLN, dissolved in 150 ml of water, regardless of individual weight. Thus, a 60 kg patient received a daily dose of GLN corresponding to 0.25g/kg/day. To obtain protective effects in ischemia / reperfusion, higher doses were used by other researchers. Alves et al.[34] used 0.50g/Kg intravenously in a single dose, over 3 hours. Even superior doses have been used in experimental studies. Todorova et al.[35] used 1g/kg/day in a rat model rat to obtain protective effects.

Safety of GLN use in human beings has been studied. Garlick[36] reported that no adverse effects of glutamine have been demonstrated when given in doses of 0.57g/kg/day in various clinical studies offering glutamine over 4 hours to 30 days. The same author concludes that no adverse effects of glutamine were found when glutamine was administered in doses of 50-60g/day. However, this assessment, made in short-term studies in hospital patients, may not be appropriate for chronic supplementation in Ca bearing subjects.

Conclusion

In the present study, oral GLN (15g/kg/day) offered no protection against systemic or local oxidative stress in women with breast Ca, undergoing neoadjuvant chemotherapy with FAC regimen.

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