Effects of physical activity on systemic oxidative/DNA status in breast cancer survivors

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Abstract. Physical activity offers a paradoxical hormetic effect and a health benefit to cancer survivors; however, the biochemical mechanisms have not been entirely elucidated. Despite the well-documented evidence implicating oxidative stress in breast cancer, the association between health benefits and redox status has not been investigated in survivors who participate in dragon boating. The present study investigated the plasmatic systemic oxidative status (SOS) in breast cancer survivors involved in two distinct physical training exercises. A total of 75 breast cancer survivors were allocated to one of three groups: Control (resting), dragon boat racing and walking group; the latter is a type of aerobic conditioning exercise often advised to cancer patients. Various biochemical oxidative stress markers were examined, including oxidant status (hydroperoxide levels, lipid oxidation) and antioxidant status (enzymatic activities of superoxide dismutase and glutathione peroxidase, reduced glutathione levels and antioxidant capability). In addition, the individual DNA fragmentation and DNA repair capability of nucleotide excision repair (NER) systems were examined by comet assays. According to the results, all patients exhibited high levels of oxidative stress. Physical activity maintained this oxidative stress condition but simultaneously had a positive influence on the antioxidant component of the SOS, particularly in the dragon boating group. DNA fragmentation, according to the levels of single- and double-strand breaks, were within the normal range in the two survivor groups that were involved in training activities. Radiation-induced damage was not completely recognised or repaired by NER systems in any of the patients, probably due to radiosensitivity and/or susceptibility of patients to cancer. These findings suggest that physical activity, particularly dragon boat racing, that modulates SOS and DNA repair capability could represent a strategy for enhancing the quality of life and improving the long-term health benefits for breast cancer survivors.

Introduction

Breast cancer is a leading cause of cancer-associated mortalities among women worldwide. Risk factors, in addition to familial history and chromosomal instability, include diet and lifestyle, high body weight, oral contraception, age at menarche/ menopause/first pregnancy, and oestrogen treatment (1). Previous data supports a causative role of oxidative stress in breast cancer development and a paradoxical effect elicited by physical activity, which increases the production of reactive oxygen species (ROS), as well as increasing antioxidant capabilities in order to counteract subsequent oxidative insults (2). ROS are dual-faceted molecules. Whereas modest levels are useful as cell signalling molecules in various physiological cellular processes, including proliferation, apoptosis, differentiation, migration, invasion and angiogenesis (3), high levels cause severe oxidative damage to cell components, such as lipids, proteins and DNA (4,5).

Antioxidants counteract increases in the production of free radicals, protect the body from oxidative damage by maintaining redox balance, and are critical for preserving optimum health and well-being. Antioxidant defence mechanisms may be enzymatic [superoxide dismutase (SOD), catalase, glutathione peroxidase (GPxs)] or non-enzymatic (vitamins and/or certain polyphenol molecules introduced through diet); for example, reduced glutathione (GSH) is particularly able to scavenge hydrogen peroxide. When ROS production occurs in the absence of sufficient defence mechanisms, a number of harmful genomic modifications may occur, including chromosomal instability, permanent DNA damage and acquisition of mutations, which may also be disposed by alterations in DNA repair systems; this can contribute to the development of various diseases, including carcinogenesis (6).

Numerous studies have indicated that upper body exercise programs, and particularly dragon boat racing, in association with cancer therapy (7) may confer benefits to cancer patients, including improving emotional and physical functioning,
increasing treatment-induced symptoms (such as nausea, fatigue or pain) and problems associated with lymphedema (8), and improving survival and quality of life. However, little is known about the biochemical/clinical factors underlying such health benefits, such as the improvement in oxidative stress pathways induced by physical activity (9,10). To date, to the best of our knowledge, no studies of the association between dragon boat racing and oxidative stress have been conducted.

Considering the contribution of regular exercise to ROS production and the subsequent biological adaptation by enhanced antioxidant enzymatic capacity (11), the present study focused on the possible association between redox status and physical activity by examining oxidant and antioxidant biomarkers in 75 breast cancer survivors involved in one of two training programs [dragon boat racing (n=25) or walking (n=25)] or at rest (n=25). Walking as well as jogging, cycling and swimming are examples of aerobic conditioning exercise that the doctors advise to cancer patients as they may enhance physical well-being and improve recovery, in addition to enhancing cardiovascular fitness and effective weight management, all of which are beneficial to individuals with lymphedema (12).

In addition, in order to evidence a possible link between breast cancer susceptibility and DNA instability, individual DNA fragmentation and nucleotide excision repair (NER) DNA repair system capability were examined by comet assay.

Materials and methods

Experimental design and subjects. Breast cancer patients (n=75; aged 35-65 years) were enrolled at the Department of Surgery of University of Catania (Catania, Italy). Additionally, healthy women (n=30; aged 40-59 years) were evaluated as control group. The characteristics of the population studied, including body mass index (BMI), age, height, weight and maximal oxygen consumption (VO

2

max) are reported in Table I. Smokers or patients with diabetes mellitus, liver disease, thyroid disease, nephrotic syndrome, hypertension and rheumatoid arthritis were not included in the study.

At ~1 month after commencing their individual therapeutic protocols, the cancer patients were separated into three groups depending on their freely chosen physical activity program (twice per week for ≥7 months) or no activity, as follows: Dragon boat racing (Group A, n=25), walking (Group B, n=25) and at rest (Control BrC, n=25). In the present experimental design the walking exercise consisted of walking briskly outdoors for 3-4 h a week along freely chosen paths.

All patients followed a controlled fruit/vegetable-rich diet. The patients were examined at different time points: i) before surgical treatment (BST); ii) after surgical treatment (AST); and iii) within 3 days after training.

The research protocol was granted ethical approval by the Hospital Committee for Research on Human Subjects and the written informed consent was obtained from each patient.

Blood samples. Blood samples obtained by venepuncture were centrifuged to collect either plasma (10 min at 800 x g) or lymphocytes by the Ficoll-Hypaque density gradient centrifugation method, as described below.

Derivatives of reactive oxygen metabolites (d-ROMs) test. Plasma samples (10 µl) were utilised to determine ROS levels by colorimetric d-ROMs test at a wavelength (λ) of 505 nm, according to the manufacturer’s protocol (Diacon International srl, Grosseto, Italy). Colour intensity was expressed in Carratelli Units (CARR U), with 1 CARR U corresponding to 0.8 mg/l of hydrogen peroxide (13). Reference values of healthy subjects are 250-300 CARR U, while high/very high levels are in the range of 401-450 CARR U or >500 CARR U, respectively.

Biological plasmatic antioxidant potential (BAP) test. Individual antioxidant power was evaluated by measuring BAP. The BAP test (Diacon International srl) spectrophotometrically (λ=504 nm) measures the capacity of the plasma to reduce iron from the ferric form (Fe

3+

) to the ferrous form (Fe

2+

) (14). The results are expressed in µmol/l; reference values of healthy subjects are considered to be ≥2,200 µmol/l.

Determination of lipid hydroperoxides (LPO). Plasma LPO levels were evaluated by a modified ferrous oxidation/xenolen orange assay, at λ=560 nm, as described by Di Giacomo et al (15). The absorbance, measured by a Hitachi U-2000 spectrophotometer, was expressed as nmol/ml plasma using hydrogen peroxide (0.2-20 µM) for calibration.

Total plasmatic thiol groups. Plasmatic thiol groups, containing predominantly reduced GSH, were determined spectrophotometrically at λ=412 nm by Ellman’s reagent [acid, 5,5’-dithiobis-(2-nitrobenzoic acid)] (15). Results are expressed as µmol/ml of plasma.

Analysis of GPx activity. The analysis of plasmatic GPx was performed using a Glutathione Peroxidase Assay Kit, according to manufacturer’s protocol (Cayman Chemical Company, Ann Arbor, MI, USA; item no. 703102), which refers to the Paglia and Valentine method (16). GPx activity was indirectly measured by a decrease in absorbance at λ=340 nm (A

340

) due to the oxidation of NADPH to NADP. Under conditions in which GPx activity is limiting, the rate of decrease in A

340

 is directly proportional to GPx activity in the sample, expressed as nmol/min/ml.

Analysis of SOD activity. The activity of all three types of SOD (Cu/Zn-, Mn- and Fe-SOD) was measured in plasma samples using a Superoxide Dismutase Assay Kit, according to the manufacturer’s protocol (Cayman Chemical Company; item no. 706002), at 440-460 nm and expressed as U/ml; 1 unit of SOD is defined as the amount of enzyme required to have 50% conversion of the superoxide radical into molecular oxygen and hydrogen peroxide (17).

Alkaline and neutral comet assay. The alkaline and neutral Comet assay protocol was performed as previously described by Tomasello et al (18). Triplicate samples (each 40 µl) mixed with 0.5% low-melting point agarose were spread on FLARE™ Slides ( Trevigen, Inc., Gaithersburg, MD, USA), immersed in cold lysis solution for 1 h, and electrophoresed for 20 min in alkaline (pH 13; 0.7 V/cm) or neutral buffer (pH 8; 0.5 V/cm). Following electrophoresis, slides were neutralised, dehydrated
by immersion in 70% ethanol and stained with SYBR Green. Analysis was conducted using an epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) with Casp Comet Assay Software (version 1.2.2; CASPLab, University of Wrocław, Poland), by measuring DNA damage as the percent of DNA in the comet Tail (% TDNA). Each phase of the procedure was performed according to European Standards Committee on Oxidative DNA Damage guidelines (19). A CometAssay® Control Cell population from Trevigen, Inc. was co-electrophoresed.

**Human umbilical vein endothelial cell (HUVEC) cultures, isolation of lymphocytes and DNA repair assay.** Ultraviolet C (UVC)-induced cell damage and the efficacy of lymphocytes extracted from cancer survivors were tested on agarose-embedded HUVECs (Thermo Fisher Scientific, Inc., Waltham, MA, USA), cultured according to Russo et al (20). Confluent HUVECs were detached with trypsin-ethylenediaminetetraacetic acid, and the number of viable cells/ml was determined by trypsin blue staining; only plates with total viable cells ≥70% were used for the NER Comet test, according to method proposed by Collins et al (21) and modified by Gaivão et al (22). The individual capability for NER of DNA from HUVECs damaged by UVC irradiation was measured to evaluating the activity of repair enzymes present in individual lymphocyte samples. Lymphocytes were isolated from heparinised venous blood on Ficoll-Hypaque gradients by centrifugation; lymphocytes extracted from each patient were prepared as described by Gaivão et al (22). Agarose-embedded HUVECs were irradiated with 1 Jm⁻² UVC on ice; this creates pyrimidine dimers and 6,4-photoproducts, which are repaired by NER. Subsequently, the contents of the slides were lysed (pH 10.0-10.5; 2.5 M NaCl, 100 mM Na₂ EDTA, 10 mM Tris and 1% lauroyl sarcosine, 1% Triton X-100 and 10% dimethyl sulfoxide were added directly prior to use) and 1% lauroyl sarcosine, 1% Triton X-100 and 10% dimethyl sulfoxide were added directly prior to use) and incubated for 30 min in a humidified chamber at 37°C. Reaction buffer alone was used as a negative control, and T4 endonuclease V (Trevigen, Inc.) was used as a positive control. Following incubation, the slides were processed according to the standard protocol for the alkaline comet assay to measure the DNA breaks introduced by the initial incision events of repair. The strand breaks produced were detected by comet assay; the increase in % tail DNA over time reflects the DNA repair activity of the cell extract.

**Statistical analysis.** The data is presented as the mean ± standard deviation in the tables and as median with interquartile range in the figures. The differences among the control groups and groups A and B were analysed by one-way analysis of variance. Statistical analysis was conducted using GraphPad Prism 5 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Boxplots were used to represent data and the differences between the individual groups were assessed with Bonferroni’s post hoc test, with P<0.05 considered to indicate a statistically significant difference.

**Results**

**Determination of SOS.** The levels of oxidative and antioxidative parameters in the plasma of the subjects prior to training activity are shown in Table II. d-ROMs levels, a measure of oxidative status, differed significantly between the groups, being higher in the BST group (500±50 CARR U) compared with the control values of healthy women (265±45 CARR U). The AST measures were significantly lower than those observed BST (420±45 CARR U). In parallel, BAP levels, a measure of antioxidative status, were significantly lower compared with the control values in cancer patients (BST and AST patients).

In order to verify a possible link between oxidative stress conditions and physical activity in cancer, women affected by breast cancer were assessed in three groups: Group A (dragon boat racing), Group B (walking) and Control BrC (breast cancer survivors at rest). The following results regarding the SOS assessed are represented by boxplots, where the range of normal values derived from the healthy control group (n=30) are indicated by the thick black line on the y-axis of each graph.

As shown in Fig. 1A, the levels of radical species were above the normal range of values; indeed, the majority of the examined subjects were in the high or very high range (between 400 and 550 CARR U). In particularly, ROS were induced by the two physical activities: 459±61 CARR U for Group A and 502±76 CARR U for Group B; however, this difference was not statistically significant (P=0.332). The increase in ROS was significant with respect to the Control BrC group for each of the two activity groups (P=0.038 and P<0.001, respectively).

As presented in Fig. 1B, the evaluation of LPO revealed higher levels in the three groups of cancer survivors compared with the healthy control values (4.22±0.064 nmol/ml; n=30). The average values for Groups A and B were 13.2±3.6 and 15.08±2.7 nmol/ml, respectively; the difference between these two activity groups was not statistically significant (P=0.224). Conversely, the differences between each of the physical activity groups and the Control BrC group (9.7±2.5 nmol/ml) were statistically significant (P=0.007 and P<0.001, respectively).

BAP data revealed that the majority of the subjects examined had a high plasmatic antioxidant potential (2,275±337 and 2,236±223 µmol/l for Groups A and B, respectively, without a significant difference between the physical activity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy control subjects (n=30)</th>
<th>Breast cancer patients (n=75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49±9</td>
<td>51±12</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>160±5</td>
<td>164±7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70±5</td>
<td>68±4</td>
</tr>
<tr>
<td>Body mass index</td>
<td>24±3</td>
<td>23±5</td>
</tr>
<tr>
<td>Maximal oxygen consumption</td>
<td>40±3</td>
<td>42±5</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation.
Additionally, following physical training, BAP levels were significantly increased compared with pre-exercise basal levels (2,000±100 µmol/l, refer to AST values in Table II) and control BrC levels.

In addition, as shown in Fig. 1D, the total plasmatic thiol levels all three cancer survivor groups overlapped with the range of values from control group (0.4-0.6 µmol/ml), and no statistically significant differences were identified between
the two physical activity groups (P=0.173). However, the thiol levels in a proportion of the women in Group B and Control BrC were below the levels of the control. The Control BrC values were also significantly lower than those of the two physical activity groups (both P<0.001).

The estimated SOD activity in each of the cancer survivor groups (Fig. 1E), was markedly higher than control values (1.82±0.039 U/ml). In particular, the activity of this enzyme was statistically significantly higher in Group B compared with that in Group A (8.4±1.9 Vs. 6.8±2 U/ml; P=0.044), as well as compared with that in the Control BrC group, which was the lowest (3.90±2.04, both P<0.001).

GPx activity levels were significantly higher in the two physical activity groups relative to the healthy control values (113.41±20.77 nmol/min/ml, both P<0.001) and the Control BrC group (147.10±37.6 nmol/min/ml, P=0.007 for Group A and P=0.007 for Group B). In particular, the level in Group A was 246±57.7 nmol/min/ml, while in Group B a slightly lower mean of 197±53.3 nmol/min/ml was observed (Fig. 1F).

DNA damage and DNA repair capability. The results regarding DNA fragmentation, measured with alkaline and neutral versions of the comet assay, are presented as % TDNA in Fig. 2A and B. The alkaline comet assay data revealed that DNA damage was higher in Group B (17.10%) compared with in Group A (14.05%), and the damage in each of these two groups was lower than in the Control BrC group (19.59%). However no statistical significance was observed among the groups. Additionally, the majority of subjects had % TDNA values within the range considered ‘normal’ for the comet assay (22). Conversely, very little double-strand break DNA damage was indicated by the neutral comet assay analysis for all groups, as shown in Fig. 2B.

The NER analysis revealed no significant difference in % TDNA between the two physical activity groups (Group A, 31.5±7.6 Vs. Group B, 30.3±8.4%; P=0.80); however, the individual repair capabilities were below that calculated with T4 endonuclease V, which was used as the reference value in the present study (% TDNA, 70%; dotted line in Fig. 3). The two physical activity groups exhibited significantly greater repair capabilities compared with the Control BrC group (24.5±6%) following UVC-induced damage (Group A, P=0.008; Group B, P=0.045).

Discussion

One adverse outcome of surgical or radiologic breast cancer treatment is the risk of lymphedema. Onset may occur months or years following treatment for breast cancer; survivors remain at risk for life so to prevent postoperative development, vigorous repetitive movements of the upper limbs was strongly discouraged until ~10 years ago (23,24). However, more recently doctors have encouraged the practice of physical activity at least six months post-treatment, for blocking lymphedema development, in addition to beneficial effects that the practice of stable and diversified physical activity exhibits in preventing carcinogenesis, in ameliorating various symptoms associated with chemotherapeutic treatment, improving the quality of life and resilience of survivors, and decreasing levels of distress (24,25). However, to the best of our knowledge, no useful data have been reported regarding the biochemical changes that underlie the improved health of cancer survivors who participate in upper body exercise programs such as dragon boating.

Numerous previous studies have documented an interference in ‘redox regulation’ associated with carcinogenesis, tumour progression and/or chemotherapeutic efficacy, including in breast cancer (2,26). In order to investigate the association between physical activity and oxidative stress-related biochemical parameters in breast cancer survivors, the present study assessed two groups of breast cancer patients involved in different physical activities twice per week: dragon boat racing (Group A) and walking (Group B).

Current opinions regarding the various indices that may be used to measure the oxidative status of patients are controversial. Such indices include total antioxidant capability (TAS or BAP test), total oxidative status (TOS), and the TAS/TOS ratio, which is expressed as the oxidative stress index. The latter of these indices is considered as the best approach for determining the net oxidative stress condition, at a diagnostic and/or therapeutic level (27).

In the present study, various oxidative stress biomarkers were evaluated. First, measurements of ROS levels (d-ROMs...
Nucleotide excision repair capability in breast cancer survivors. Naked DNA from ultraviolet C-irradiated human umbilical vein endothelial cells were incubated in gel with buffer, or T4 endonuclease V, or lymphocyte extracts from each subject. Following 45 min of incubation, DNA breaks introduced by repair endonuclease activity in the lymphocyte extracts were measured by comet assay and expressed as % TDNA, i.e. the percentage of DNA in the comet tail. For each group, the line in the middle of the box represents the median, the black dash represents the mean value, and the lower and upper edges of the box represent the 1st and 3rd quartiles, respectively. Whiskers represent the minimum and maximum values; observations denoted as black circles are considered outliers. The dotted line represents the calculated % TDNA (percentage of DNA in the comet tail) from T4 endonuclease V, which was considered as reference value for evaluating patients repair capability. *P<0.01; **P<0.05. Group A, dragon boat racing group; Group B, walking group; Control BrC, breast cancer survivors at rest.

Test, which is similar to TOS) and antioxidant capability (BAP test, which is similar to TAS) were taken in patient groups BST and AST. Secondly, following the physical training programs, in addition to the aforementioned parameters, the plasmatic LPO and GSH levels and the enzymatic activities of SOD and GPx were examined in the breast cancer survivors. Furthermore, DNA status and repair capability in the experimental groups were explored. The present data demonstrated that enhanced oxidative stress was present at time of diagnosis in all enrolled subjects, which is in agreement with data from a number of previous studies (28,29), affecting both components of SOS. Certain authors have reported that the activities of all the studied antioxidant enzymes (SOD, catalase, GPx and glutathione S-transferases) and the levels of reduced GSH were significantly increased in breast cancer patients compared with their healthy control group (30); whereas others have reported reduced SOD and GPx activities in breast cancer patients (31). In the present study, the oxidative stress level was improved marginally following surgical treatment, and was positively affected by physical activities, but in different ways in the two survivor groups who undertook training programs. Enzymatic and non-enzymatic antioxidants, as well as lipid peroxidation, have been reported to be altered among various tissues types and individual breast cancer patients (32,33). In the present study, in the presence of elevated levels of ROS (based on the d-ROMs test), high levels of LPO levels were observed following physical training. This ROS increase positively influences the plasmatic antioxidant component (BAP), and may be considered a direct reaction to a physical activity-induced oxidative environment.

In the present study, despite the high ROS production, the total plasmatic thiol group levels, comprising mainly GSH, were not depleted but were maintained within the reference normal range, being higher in Group A than in Group B. This data demonstrates the benefits of physical activity, as previously reported in other studies (34,35). We consider that the high level of GSH, as well as the GPx and SOD activity, may be generated to counteract the effects of increased oxidative stress and lipoperoxidation, as an adaptive response to the increase in circulating ROS; this was also reported by Carter et al (36). Such an adaptive response, mediated by a physical activity-induced oxidative environment, may involve changes in antioxidant gene expression via the antioxidant-responsive elements, which may be consistent with the enhanced enzyme activities observed as previously reported by Kobayashi et al (37). Furthermore, in our opinion, the maintenance of high antioxidant levels observed in the studied patients is the result of the diet rich in vegetables/fruit and the physical activity, as reported in a previous study (38).

Certain previous studies have reported the presence of higher baseline DNA damage in breast cancer patients without physical training (39,40). The present results regarding double-strand break DNA damage (neutral comet assay) indicated that the % TDNA values were similar in all breast cancer patients and were comparable with healthy control values. A similar finding was observed in the majority of women in both groups that underwent training programs when using the alkaline comet assay, although some patients exhibited values higher than that of the reference control. These data may be associated with possible persistent DNA damage and non-functional/inefficient DNA repair systems.

It is well-known that most damage is removed by repair enzymes before it is able to interfere with DNA replication and introduce mutations. Individual variation in DNA repair capacity is therefore likely to be an important factor in determining cancer risk. In the present study, the DNA-NER capability, represented by the activity of lymphocyte extracts, was less than the T4 enzyme-treated control in all of the studied women. In particular, the specific lesions that occur following UVC radiation exposure, which are recognised and excised by T4 endonuclease (1 unit), produced an increase in % TDNA equal to 70% over time, reflecting the maximum DNA repair activity. By contrast, the lymphocyte extract of the breast cancer patients exhibited repair capacities equal to 31.04 and 30.15% for Groups A and B, respectively. This data demonstrates that almost half of radiation-induced damage was not repaired by the enzyme activity of the lymphocyte extracts, and remained as persistent lesions. Shahidi et al (41), who used a kinetic repair approach, demonstrated the radiation-induced DNA damage is not completely repaired compared with control subjects after 3 h, leading us to hypothesise that deficient radio-induced damage repair may promote the onset of late harmful irradiation effects in breast cancer patients.

One limitation of this in vitro assay is that the lymphocytes are not directly irradiated, and the NER system is perhaps not sufficiently activated, which may have produced the deficient repair activity observed. Similarly to Gaivão et al (22), who performed an NER-assay on healthy subjects, inter-individual variability of repair activity in cancer survivors was observed in the present study; this may arise from genetic polymorphisms and epigenetic factors,
thus influencing individual susceptibility to cancer development. In our opinion, the present data is of interest, although the experimental approach must be repeated and extended to a larger number of samples, particularly because this data may be useful in the context of individual radiosensitivity in women with breast cancer for whom radiotherapy is chosen as a treatment. Considering this data on DNA repair, we hypothesise that dragon boating and continuity of physical activity, inducing ROS production, may stimulate an increase in DNA repair ability over time, among the possible adaptive responses; this is also in agreement with Mao et al (42). In conclusion, the present data indicates that the measurement of different blood redox biomarkers may be a useful approach in defining an individual antioxidant therapy to support and/or reinforce the efficacy of primary treatments; that the monitoring of DNA repair capacity (in particular the NER system) may be useful in defining an eventual radiotherapeutic plan; and that dragon boating is beneficial for breast cancer survivors, leading us to suggest the large scale adoption of this activity, since it may also lead to considerable savings in costs associated with physiotherapy.

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References


