Exercise Training-induced Modulation in Microenvironment of Rat Mammary Neoplasms

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ABSTRACT
Despite the importance attributed to exercise training in the breast cancer (BC) continuum, the underlying mechanisms modulating tumor behavior are unknown. We evaluated the effects of long-term moderate-exercise in the development of mammary tumors, and studied the microenvironment of infiltrative lesions, the amount of connective tissue, and balance between cellular proliferation/death.

Fifty Sprague-Dawley rats, randomly assigned into four groups: two control groups (sedentary and exercised) and two models of BC groups (sedentary and exercised) induced by N-methyl-N-nitrosourea (MNU), were sacrificed after 35 weeks of moderate-exercise, and all perceptible tumors were removed for histological and immunohistochemistry analysis.

The median number of infiltrative-lesions per animal was lower in the MNU exercised animals (p = 0.02). More than one histological pattern was identified, and papillary carcinoma was the most frequent in both groups. Within infiltrative-lesions, the number of immunopositive cells per μm² of Ki67 was lower in exercised animals (p = 0.002). This presents increased cell death per μm² (p = 0.019). Tumors from sedentary animals had a higher expression of collagen deposition (p = 0.027).

Long-term moderate-exercise has beneficial effects in tumor development with a diminished prevalence of malignancy. Within infiltrative-lesions, moderate-exercise improves the balance between cell-proliferation and cell-death with decreased connective tissue that suggests lower tumor aggressiveness.
The proliferative capacity of a tumor is one of the most important variables that determine tumors’ progression along with its ability to undergo apoptosis [8, 31]. In fact, what defines the growth rate of a given cell population is the balance between cellular proliferation and death. Thus, improve the competency of the apoptotic machinery in malignancy is an important target in breast cancer [10]. Furthermore, a relationship is established between malignant cells and the TME during cancer progression. This is composed of the extracellular matrix (ECM) and cellular components. Moreover, it is now well-documented that neoplastic cells are influenced by the surrounding microenvironment and vice-versa [37]. The crosstalk between cancer cells and the other TME-associated cells (e.g., fibroblasts and macrophages) may underlie the tumor’s capability to grow and metastasize [22].

It is unclear how mammary tumors in animals respond to exercise training. The few research reports in this area show promising results by associating exercise training exposure with reductions in proliferation-associated proteins together with increased expression of apoptosis-associated proteins, but this needs confirmation [25–27, 48]. It remains unknown if exercise training modulates neoplastic stroma.

Here, we used a well-characterized model of breast cancer induced in rats by N-methyl-N-nitrosourea (MNU) to determine the long-term exercise effects in the microenvironment of neoplastic tissue. Previously, our research team has shown that exercise training can reduce malignancy [14]. Here, we hypothesized that exercise training could favorably modulate the TME via the following: (1) reducing their proliferative capacity; (2) improving their ability to undergo cell death; and (3) remodeling the TME stroma.

Materials and Methods

Animals

Fifty female Sprague-Dawley rats (38 days old; 289 ± 17 g body weight) from Harlan Interfauna Inc. (Barcelona, Spain) were randomly housed in collective cages (4 animals per cage) and maintained under controlled atmospheric conditions (21–22 °C; 60 ± 5 % humidity) in a 12/12 h light/dark cycle with free access to food (standard laboratory diet 4RF21® Mucedola, Italy) and water. All the ethical principles of research involving animals were met [23]. The Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Alimentação e Veterinária) approved the animal protocol with license number 008961, and the experimental protocol was performed in accordance to European Commission Recommendation 2007/5266/CE.

Experimental design

After two weeks of acclimatization, the animals were randomly assigned to one of four groups: sedentary injected with MNU (MNU + SED, n = 15); exercised injected with MNU (MNU + EX, n = 15); sedentary control, injected with sterile saline solution (CONT + SED, n = 10); and exercised control, injected with sterile saline solution (CONT + EX, n = 10). Animals from the MNU groups were injected intra peritoneally (i.p.) with 1-methyl-1-nitrosourea (ISONARC®, Sigma Chemical Co., Madrid, Spain) given in a dose of 50 mg/kg; the control groups received i.p. injection of vehicle (sterile saline solution 0.9%). Two days after carcinogen injection, animals from the exercised groups started a treadmill running program (Treadmill Control LE 8710, Harvard Apparatus, USA) for 35 weeks. The duration and intensity of running were gradually increased over the first two weeks until it reached 60 m/day, 5 days/week and 20 m/min of speed (estimated work rate of 70 % maximum oxygen consumption) [32]. This was maintained until the end of the experimental protocol. Sedentary animals were manipulated daily to establish identical conditions to active animals.

At the end of experimental protocol, animals were euthanized by i.p. injection of ketamine (75 mg/kg, Imalgen® 1000, Merial SAS, Lyon, France) and xylazine (10 mg/Kg, Rompun® 2 %, Bayer Healthcare S. A., Kiel, Germany). In each animal, all perceptible tumors were removed, counted, weighed, and prepared for histological and immunohistochemical analysis.

Histological and immunohistochemical analysis

Briefly, samples were fixed with paraformaldehyde, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Paraffin blocks were then cut into 5μm sections with a Leica 2125 rotary microtome (Leica Microsystems Inc.). The paraffin embedded slides were deparaffinized in xylene and hydrated using a series of graded ethanol. They were stained with hematoxylin and eosin (H&E) for histological classification and with Picosiris Red (PSR) to assess collagen content (CC). Tumors were classified according to Russo and Russo [38] and presented different histological patterns (here designated as lesions). After being identified and quantified, all infiltrative lesions were further analyzed via the TUNEL assay and Ki67 immunostaining.

TUNEL assay

A TUNEL test (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) was performed (In Situ Cell Death Detection Kit, Ap, 11684809910 from Roche®, Germany) according to manufacturer’s instruction on paraffin embedded tissues using the citrate buffer (pH = 6.0). Samples were then heat-treated (in a pressure cooker) for 5 min for antigen retrieval. Appropriate positive and negative controls were used throughout. After Fast Red application, the slides were rinsed in water, counterstained with hematoxylin for 5 min, and mounted with Crystal Mount (aqueous-based). Imaging detected and quantified the cell death per μm².

Ki67 immunostaining

Rabbit monoclonal [SP6] antibody to Ki67 (rabbit ab16667 from Abcam®, England) was used to quantify cells into cell cycle per μm². Slides were immersed in citrate buffer (pH = 6.0) and heat-treated (in a pressure cooker) for antigen retrieval for 15 min. This was then cooled down in citrate buffer for 30 min. Slides were rinsed in PBS (pH = 7.4) twice between applications of each of the following reagents. Endogenous peroxidase activity was blocked applying a solution with methanol, hydrogen peroxide, and PBS-Tween (phosphate-buffered saline) for 30 min. Bovine serum albumin (3 %) was applied 30 min for non-specific blocking. Primary and secondary (goat anti-rabbit HRP, ab97069 from Abcam®, England; HRP-conjugated secondary antibody) antibodies were applied for 120 min and 60 min, respectively, in a humidified 37 °C chamber. After DAB (diaminobenzidine) application for 7 min, the slides were rinsed in water, counterstained with methyl-green for 3 min, dehy-
drated using a series of graded ethanol, cleared in xylene, and mounted with DPX (Distyrene Plasticizer Xylene). Negative controls for primary and secondary antibodies were used throughout.

After all of these procedures, slides from each malignant lesion were scanned using a Virtual Slide System VS110 (Olympus, Japan), and the digital slides were analyzed using Fiji ImageJ Pro and Image Pro (Media Cybernetics, version 6.0). Several parameters were assessed from each analyzed section including: (1) the number of Ki67-positive nuclei per μm² [5]; (2) the number of TUNEL-positive nuclei per μm² [11] and; (3) the overall percentage of collagen within the tissue section [24].

**Statistical analysis**

All quantitative variables were expressed as median and percentiles (25th and 75th) due to their abnormal distribution as previously assessed with the Shapiro-Wilk test. All categorical variables were expressed as an absolute and/or relative frequency. For quantitative variables, data comparison between groups was done with a non-parametric Mann-Whitney test one-tailed; inter-group comparison of categorical variables was performed with the Chi squared test. The odds ratio (OR) with 95% CIs were also calculated (Fisher’s exact test) to establish the association between sedentary and exercise behaviors with the odds of developing an infiltrative lesion. A p value < 0.05 was considered statistically significant. Graph Pad Prism software (version 7.0) was used for all analysis.

**Results**

All MNU animals developed tumors at the end of the experimental protocol; no tumors were detected in the control groups. The number of tumors developed was similar in both groups [14]. No significant differences were found in animals’ weight and the same happen regarding the amount of food intake [36].

*Table 1* shows no significant differences between groups in the infiltrative tumor’s total weight. Non-infiltrative lesions were slightly higher in the MNU sedentary animals, but this was not significant. However, the positive impact of exercise training can be observed in the prevalence of infiltrative lesions per animal—these were significantly lower in the exercised MNU animals (p = 0.020) [14].

The frequency of distribution for the different types of lesions between groups (*Fig. 1*) was significantly different (p = 0.0005). Different histological patterns of non-infiltrative (*Fig. 1a*) and infiltrative (*Fig. 1b*) lesions were identified. The most frequent infiltrative type for both groups, although lower in exercised animals, was the papillary type followed by the cribriform pattern. No invasive comedocarcinoma pattern was detected in MNU-exercised animals. This was the most aggressive lesion identified in MNU sedentary animals. Similar rates of pre-neoplastic lesions (intraductal proliferation) were found in both MNU groups.

Additionally, the computed OR revealed that the odds of appearance of an infiltrative lesion in sedentary animals are higher (OR = 1.27; CI: 0.67–2.37) than the odds of this appearance in exercised animals. The opposite is seen relative to non-infiltrative lesions (OR = 0.79; CI: 0.41, 1.51). These are less likely to appear in MNU sedentary animals. This means that the probability of a MNU sedentary animal developing an infiltrative lesion is 27 % higher.

**Immunohistochemical analysis**

The positive influence exerted by long-term (35 wks.) moderate exercise training in malignant lesions was noted in their density of cell proliferation and cell death. Cell proliferation was evaluated by Ki67 staining by counting the positive nuclei in all sections of the scanned slides (*Fig. 2a, b*). An increase in cell proliferation (322...
(265.5–442.5]) was observed in sedentary animals comparatively to exercised animals (194 [98.5–284.5]; ➤ Fig. 2c).

Cell death was assessed by TUNEL staining, and the positive nuclei for DNA fragmentation were counted using all sections of the scanned slides. ➤ Fig. 3 shows the collected data for cellular death fraction—a decrease in positive nuclei (19 [15.75–39.5]) can be observed in MNU sedentary (➤ Fig. 3a) animals compared to the MNU exercised animals (➤ Fig. 3b, 38 [27.5–70.5]). Significant differences were observed between MNU groups (➤ Fig 3c).

**Collagen content analysis**

The collagen content was measured as the percentage of pixels containing the red signaling in tissue images. Exercised animals (➤ Fig. 4b; 6.16 [3.76–9.48]) had a lower amount of connective tissue in the TME when compared with sedentary animals (➤ Fig. 4a, 7.41 [4.97–10.22]) (➤ Fig. 4c).

**Discussion**

This study provides strong evidence for the protective effects of exercise training on tumor development and malignancy accompanied by substantial improvements in the features of the lesions. In contrast to previous reports that associated a reduction in tumor burden with exercise training, we found that breast tumors grew at comparable rates in sedentary and exercising animals [3, 19, 25–27, 33, 35, 44–47, 49, 50]. Other researchers have reported that the increased rates of mammary tumor growth are associated with exercise exposure [6, 7, 14, 33]. These contrasting results can be explained by differences in the protocols.

For example, Zhu et al., Jiang et al., Goh et al., and Welsch et al., found positive results in tumor size associated with voluntary exercise, but the duration of the experiments ranged from 5 to 10 weeks [19, 26, 27, 44, 49, 50]. Only one report has associated voluntary exercise with a 40.0% decrease in tumor size, but this used a longer protocol (20 weeks) [41]. In contrast, Cohen et al., and Colbert et al., found the opposite results in animals subjected to voluntary exercise for a longer period (20 and 21 weeks respectively) [6, 7].

If we consider forced exercise designs, then we can verify the same tendency of exercise-induced reduction in tumor size associated with small duration experiments (between 2 and 12 weeks) [3, 33, 45, 47]. Exercise-induced tumor growth appears to be associated with longer experiments (20 and 35 weeks) under forced exercise designs [7, 14]. These differences might be partially explained in voluntary exercise experiments. There is an inevitable reduction in the amount of exercise performed when tumor burden increases. Whereas in forced exercise protocols like ours [15], the heavier and bigger tumors observed over longer duration experiments might result from the increased vascularization induced by exercise [28, 29]. It is also important to mention that, if these differences in exercise modalities could influence tumor burden, they also may trigger different biological effects. In fact, the benefits of regular exercise were also shown in the histology data. The tumor-bearing animals exposed to exercise training presented fewer non-infiltrative lesions and fewer infiltrative lesions. Exercise training might hamper the progression from noninvasive to invasive lesions, and this might explain, at least partially, the significant differences in the prevalence of malignant lesions. These differences toward a

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[Fig. 2](#) Representative light micrographs from malignant lesions of sedentary a and exercised animals b stained for ki67. Comparatively to A, it is notorious a lower density of brown marked nuclei in A. In C it is depicted a boxplot diagram of values observed in both groups.
**Fig. 3** Representative light micrographs from malignant lesions of sedentary a and exercised animals b stained for Tunel. Comparatively to A, it is notorious a higher density of red marked nuclei in A. In C it is depicted a boxplot diagram of values observed in both groups.

**Fig. 4** Representative light micrographs from malignant lesions of sedentary a and exercised animals b stained with Pricosirius red. Comparatively to A, it is notorious a lowers density of red stained area in A, which is indicative of a reduced collagen content. In C it is depicted a boxplot diagram of values observed in both groups.
lower aggressiveness in the histological lesions of exercised animals could be related to cancer-reduced systemic inflammation as we described previously [14]. Indeed, the levels of CRP and IL-6 were higher in sedentary animals. They also had a heavier spleen weight. This establishes an association between exercise training and decreased expression in systemic markers of inflammation that might be mediated by improvements in the host immune response, which is consistent with other results [18]. It remains unclear if the same thing happened in the TME of exercised animals once we no longer measured intratumoral markers of inflammation or immune infiltration. Obviously, the expression of immune mediators (e.g. natural killer cells and macrophages) could be activated or suppressed as could the expression of pro-inflammatory cytokines [20]. IL-6 and other factors secreted by the tumor influence the behavior of TME-associated cells such as macrophages and fibroblasts, and these factors can lead to alternative activation pathways for these cells [42]. The conversion of Type 1 macrophages (M1) into Type 2 macrophages (M2), for example, produces IL-10 and TGF-β and alters the differentiation of T cells away from the cytotoxic Th1 response. This can cause the cancer cells to attain stem cell-like features and can increase the fibrosis content [4].

The high degree of proliferation in the infiltrative lesions of sedentary animals suggests an overexpression of pro-inflammatory markers. This could suppress the anti-tumor immune response and can lead to increased malignancy [20]. Exercise could also impact absorption of MNU. Indeed, if the dose, route, and age of administration were the same in both groups, then the differences in the outcomes might depend on variations in the carcinogenic’s toxicokinetics and toxicodynamics as mediated by exercise training [1]. Interestingly, other papers do not describe differences in histology unlike our work [2]. Thus, is spite of our results being encouraging they can not be compared with others.

The reduced malignancy seen here could also be via modulation of malignant tissue properties. Obviously, uncontrolled cell proliferation and decreased apoptosis are key features of malignancy [21]. Increased proliferation and suppression of apoptosis are hallmarks of malignant tumors, and they play a central role in the development and progression of cancer [13]. Ki67 is a prognostic and predictive marker in breast cancer, and higher values of this protein are normally associated with poor prognosis [12]. Our results show that the infiltrative tumors of the animals exposed to moderate exercise training have lower Ki67 expression versus sedentary animals.

Our findings indicate that exercise training positively affected the growth fraction of malignant tissue differently than what has been previously reported by Zhu and colleagues in an identical work [48]. They found that physical activity had no significant influence on the immunoeexpression of Ki67 in mammary carcinomas Malicka and co-workers also saw no changes in the expressiveness of Ki67 [33]. On the contrary and similar to us, Isanejad and co-workers described significantly decreased expression of Ki67 protein in tumor-bearing animals subjected to interval training [25]. Other studies have analyzed different markers to evaluate the proliferation of neoplastic cells in breast tumors in animals. In general, the benefits of exercise training are associated with decreased levels of cell cycle regulatory proteins [26, 49]. We think that these conflicting results can be related to the differences in exercise design (i.e., amount of performed exercise and total experiment duration).

The increased cell survival via blocked programmed cell death is the other face of tumor development. It is well established that tumor cells can acquire resistance to apoptosis through different mechanisms including increased anti-apoptotic proteins or decreased pro-apoptotic proteins [43]. Here, lifelong moderate exercise training also impacted cell death and seems to induce a pro-apoptotic environment in infiltrative lesions of the MNU active animals. Similarly to prior work our results show that exposure to moderate exercise training increases the number of apoptotic cells in exercised animals [27, 48]. Previous reports have studied markers of apoptosis in animal models of breast cancer exposed to exercise and found that some anti-apoptotic markers (e.g. Bcl-2, Xiap – X-linked inhibitor of apoptosis pathway) are decreased, and some pro-apoptotic (e.g. Bax, caspase 3, caspase 8, Apaf-1 – apoptosis peptidase-activating factor-1) markers are overexpressed in active animals. These findings suggest that exercise training can induce apoptosis via a mitochondrial pathway (intrinsic pathway) or via a death-receptor pathway (extrinsic pathway) [26]. It remains unclear what might trigger this chain of events through different pathways. This is explained, at least in part, by the response of the host’s immune system to tumors with different histological types [22].

The lower levels of collagen in the exercised animals speaks suggests less fibrotic stimuli (such as TGF-β) and less activity of cancer-associated fibroblasts (CAFs). CAFs are known to secrete IL-6 and matrix metalloproteinase (MMP) that break down the ECM to facilitate cancer cell migration and metastasis [4]. Therefore, exercise could be an advantage because it could modulate the TME away from tumorigenesis. Furthermore, the increased deposition of collagen type I and III by CAFs can alter the ECM microenvironment. This could provide additional oncogenic signs for cancer cell proliferation [30]. The lower expression of collagen seen in our exercised animals agrees with the equally low expression of cancer cell proliferation.

**Conclusion**

The main findings of our work are that long-lasting moderate exercise training can reduce tumor progression by significantly decreased malignancy, cell proliferation, and increasing cell death, which reflect an improved tissue microenvironment. There is strong evidence for decreased collagen deposition. Although the biological mechanism by which these benefits were achieved are not known, these results are a valuable contribution and confirm that exercise is an important non-pharmacological approach to reduce the growth and metastatic potential of mammary neoplasms.

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**Conflict of Interest**

No conflict of interest has been declared by the author(s).
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