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Long-term exercise training as a modulator of mammary cancer vascularization

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ABSTRACT

Background: Breast cancer remains a leading cause of death by cancer worldwide. It is commonly accepted that angiogenesis and the expression of angiogenic factors such as vascular endothelial growth factor-A (VEGF-A) is associated with the increased risk of metastasis and poor patient outcome.
Objective: This work aimed to evaluate the effects of long-term exercise training on the growth and vascularization of mammary tumors in a rat model.
Materials and methods: Fifty female Sprague-Dawley rats were divided into four groups: two *N*-methyl-*N*-nitrosourea (MNU)-exposed groups (exercised and sedentary) and two control groups (exercised and sedentary). MNU was administered once, intraperitoneally at 7 weeks-old. Animals were then exercised on a treadmill for 35 weeks. Mammary tumors were evaluated using thermography, ultrasonography [Power Doppler (PDI), B Flow and contrast-enhanced ultrasound (CEUS)], and immunohistochemistry (VEGF-A).
Results: Both, MNU sedentary and exercised groups showed 100% of tumor incidence, but exercised animals showed less tumors with an increased latency period. Exercise training also enhanced VEGF-A immunoexpression and vascularization (microvessel density, MVD) ($p < 0.05$), and reduced histological aggressiveness. Ultrasound and thermal imaging analysis confirmed the enhanced vascularization of tumors on exercised animals.
Conclusion: Long-term exercise training increased VEGF-A expression, leading to enhanced tumor vascularization and reduced tumor burden, multiplicity and histological aggressiveness.

1. Introduction

Despite recent advances in diagnostic and therapeutic approaches, breast cancer remains one of the leading causes of death by cancer worldwide [1,2]. Angiogenesis, the formation of new blood vessels from pre-existing vessels and vascular endothelial cells, is essential for tumor growth, by supplying nutrients and oxygen [3,4]. Angiogenesis is regulated by a balance between proangiogenic and antiangiogenic factors, produced by both tumor and host cells, namely endothelial cells, pericytes and leokocytes [5]. Vascular endothelial growth factor (VEGF) is the most potent and widely distributed angiogenic factor [6]. The VEGF family is composed of several members – VEGF-A, –B, –C, –D and –E – of which VEGF-A is the most potent [7]. VEGF-A stimulates endothelial cell proliferation and migration, prevents the regression of newly formed vessels and increases microvascular permeability [5;6]. VEGF-A expression has been associated with cancer progression, increased risk of metastasis and poor outcome of lung, esophagus, colorectal and breast cancer [8,9]. Tumor vascularization can be non-invasively assessed by imaging tools such as ultrasonography and thermography [10]. Ultrasonography is very useful in women with dense breasts and in the characterization of breast lesions identified in mammographic examination [11], being frequently used as an adjuvant tool for

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clinical breast examination. Thermography measures the infrared radiation emitted from the body, revealing superficial temperature patterns which are directly related to local vascularization, and may therefore be used to study physiological and pathological vascular changes [12–14]. This technique was first introduced for breast cancer screening in 1956 and later recognized by the Food and Drug Administration as a tool for breast cancer risk assessment [15].

It is largely accepted that exercise training exerts a beneficial effect in some lymphomas and in colon, lung, endometrial, prostate and breast cancer [16–23]. Systematic reviews have concluded that the practice of physical activity in cancer patients improves important clinical (quality of life and fatigue) and physiological outcomes (muscle strength) [24]. Furthermore, several investigators have studied the effects of exercise training on the biopathology of mammary tumors themselves. However, these studies have focused their attention on the effects of shorter exercise training protocols using xenograft models [25–27]. Despite their usefulness and widespread application, xenograft models show important limitations, related to the lack of a functional immune system and of the complex tumor cell population which, in spontaneous tumors, evolves through a lengthy multi-step process of carcinogenesis. In particular, xenograft models are considered too artificial for studying tumor angiogenesis, and more realistic models are being called for [28]. This work intends to address these concerns, by choosing a mammary cancer model induced in immune-competent rats by *N*-methyl-*N*-nitrosourea (MNU). We hypothesized that exercise training can modulate the microenvironment of mammary tumors, and thus it was studied the effects of long-term exercise training on tumor growth and vascularization, employing thermography, ultrasonography and immunohistochemical techniques.

2. Materials and methods

2.1. Animals

Fifty female Sprague-Dawley rats, with 4–5 weeks of age were obtained from Harlan Interfauna Inc. (Barcelona, Spain). Animals were housed at the facilities of the University of Trás-os-Montes and Alto Douro in filter-capped polycarbonate cages with corncob for bedding under controlled conditions of temperature ($23 \pm 2\,^{\circ}C$), humidity ($50 \pm 10\%$), air system filtration (10–20 ventilations/hour) and on a 12 h:12 h light:dark cycle. Tap water and a basic standard laboratory diet (4RF211, Mucedola, Italy) were supplied *ad libitum* during the study. Cages were cleaned and water was changed once *per* week. All procedures were done in accordance with European and National Legislation (European Directive 2010/63/EU and National Decree-law 113/2013). The Portuguese Ethics Committee for Animal Experimentation approved all the experiments and procedures carried out on the animals (*Direcção-Geral de Alimentação e Veterinária*, Approval no. 008961).

2.2. Animal experiments

After one week of quarantine, animals were allowed to acclimate to laboratory conditions for two weeks. Then, they were randomly divided into four experimental groups: MNU sedentary (n = 15), MNU exercised (n = 15), control sedentary (n = 10) and control exercised (n = 10). The development of mammary tumors was induced in animals from both MNU sedentary and MNU exercised groups by a single intraperitoneal administration of the carcinogen agent MNU (Isopac, lot 100M1436 V, Sigma Chemical Co., Madrid, Spain) at a dose of 50 mg/kg, at seven weeks of age. MNU was used within one hour after its preparation. Animals from control groups received a single administration of the vehicle (saline solution 0.9%). After this, animals from exercised groups were acclimated to the treadmill running (Treadmill Control® LE 8710, Panlab, Harvard Apparatus, USA) for a five-day period at a speed of 20 m/min increasing progressively from 20 to 60 min/day. Then, the duration of the exercise was maintained as 60 min/day, 5 times/week during 35 weeks. The animals were daily observed to monitor their general health status. They were weekly palpated for the detection of mammary tumors development. The animals' body weight was measured weekly using a top-loading scale (Mettler PM 4000, LabWrench, Midland, Canada). The day of the MNU administration was considered the first day of the study and the animals' sacrifice 35 weeks later was considered the end of the study. At the end of the study, the accurate body weight was calculated by subtracting the tumors' weight to the total body weight, and the mortality index (MI) was calculated using the following equation:

$$MI(\%) = \frac{\text{number of animals that died during the study}}{\text{number of animals at the beginning of the study}} \times 100$$

2.3. Mammary tumors evaluation

Twenty-four hours before the examination, the skin overlying the mammary tumors was shaved using a machine clipper (AESCULAP® GT420 Isis, Aesculap Inc, Center Valley, PA, USA). At the end of the experimental protocol, immediately before the animals' sacrifice, the mammary tumors were evaluated by thermography and ultrasonography. For these examinations, all survived animals were anesthetized by intraperitoneal administration of ketamine (75 mg/kg; Imalgene 1000, lot LBF133BB, Merial S.A.S., Lyon, France) and xylazine (10 mg/kg; Rompun 2%, lot KPO78 × 0, Bayer Healthcare S.A., Kiel, Germany).

2.4. Thermographic evaluation

The thermographic evaluation was performed using a far infrared camera from FLIR® model A325 (USA), with a sensitivity of 68 mK and a spatial resolution of 320 × 240 pixels. The images were recorded at one frame *per* second for future analyses but the integration time for the micro bolometer was approximately 16.6 ms. Animals were manually held and filmed at a constant distance (0.35 m). The animal emissivity was set to 0.98 and the tumor borders were marked to overlap with a visible image [29]. Representative frames were selected and analyzed using the ThermaCam Researcher Pro 2.10 (FLIR Systems, Inc., USA) software. In this analysis, the maximum, minimum and average temperatures of each region of interest were obtained. These measurements reflect the vascularization and the extension of necrotic areas of mammary tumors. Higher vascularized tumors are expected to exhibit less extensive necrotic areas and consequently to present higher maximum, minimum and mean temperature, and lower thermal amplitude. The opposite is expected to poor vascularized tumors.

2.5. Ultrasonographic evaluation

For ultrasonographic analysis it was used a real-time scanner (Logic P6®, General Electric Healthcare, Milwaukee, WI, USA) and a 10 MHz linear transducer with a standoff pad (Sonokit1, MIUS Ltd, Gloucestershire, England). The animals were placed in supine position and it was applied acoustic gel (Aquasonic®, Parker Laboratories Inc, Fairfield, New Jersey, USA), the ultrasonographic images using Power Doppler (PDI) and B Flow modes were