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Original Article

Breast cancer pulmonary metastasis is increased in mice undertaking spontaneous physical training in the running wheel; a call for revising beneficial effects of exercise on cancer progression

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Abstract: It has been repeatedly shown that regular aerobic exercise exerts beneficial effects on incidence and progression of cancer. However, the data regarding effects of exercise on metastatic dissemination remain conflicting. Therefore, in the present study the possible preventive effects of voluntary wheel running on primary tumor growth and metastases formation in the model of spontaneous pulmonary metastasis were analyzed after orthotopic injection of 4T1 breast cancer cells into mammary fat pads of female Balb/C mice. This study identified that in the mice injected with 4T1 breast cancer cells and running on the wheels (4T1 ex) the volume and size of the primary tumor were not affected, but the number of secondary nodules formed in the lungs was significantly increased compared to their sedentary counterparts (4T1 sed). This effect was associated with decreased NO production in the isolated aorta of exercising mice (4T1 ex), suggesting deterioration of endothelial function that was associated with lower platelet count without their overactivation. This was evidenced by comparable selectin P, active GPIIb/IIIa expression, fibrinogen and vWF binding on the platelet surface. In conclusion, voluntary wheel running appeared to impair, rather than improve endothelial function, and to promote, but not decrease metastasis in the murine orthotopic model of metastatic breast cancer. These results call for revising the notion of the persistent beneficial effects of voluntary exercise on breast cancer progression, though further studies are needed to elucidate mechanisms involved in pro-metastatic effects of voluntary exercise.

Keywords: Metastasis, 4T1 breast cancer, exercise, wheel running

Introduction

Breast cancer is one of the most prevalent malignant diseases killing approximately 50,000 women worldwide each year. Although there is a large body of epidemiologic evidence suggesting reduced risk of developing the malignant disease by physically active women, the impact of physical exercise on breast cancer patient prognosis (i.e. primary tumor growth and metastatic spread) along the disease continuum is less clear [1]. Based on the literature, it has been postulated that regular physical exercise is beneficial in the treatment of various disorders ranging from psychiatric, metabolic, cardiovascular and pulmonary diseases as well as musculoskeletal disorders and, finally, cancer [2]. Indeed, regular physical exercise was shown to improve quality of life, cardiorespiratory fitness, physical functioning and fatigue of breast cancer patients and survivors [3]. It has also been shown to reduce breast cancer death rates and recurrence [4-7]. However, some controversy still exists whether exercise is always beneficial with respect to breast cancer [5, 8] since there are also reports revealing no association between physical exercise and breast cancer outcome [9, 10]. Similarly, although many pre-clinical studies performed in animal models of breast cancer

showed beneficial effects of exercise on disease progression [8, 11-13], there are also various reports indicating the lack of effect [13-15] or even increased disease incidence and worse prognosis [16].

With respect to pre-clinical studies, the differential effects of physical activity on disease progression could be attributed to the mode of exercise chosen. There is general agreement that voluntary physical activity (i.e. wheel running by rodents) reflects better natural activity patterns of animals and is associated with lower stress levels, than forced treadmill training [17]. It has certainly been demonstrated that voluntary exercise slowed down the growth of primary tumor [12, 13, 18], although it did not affect the metastatic burden [13-15], while forced treadmill exercise increased tumor multiplicity and decreased animal survival [16]. Moreover, there is general agreement that the best type of animal models to study antimetastatic effects of exercise are orthotopic models that allow for complex microenvironmental organotypical interaction between tumor cells and the surrounding stroma and that mimic primary tumor growth, differentiation, intravasation and metastatic spread in human patients [19]. Therefore, the orthotopic breast cancer model based on the injection of 4T1 cells into mammary fat pads of female Balb/C mice seemed to be the best choice to delineate the effects of exercise on breast cancer dissemination in pre-clinical studies. This model was previously used by [18] to assess the effects of voluntary exercise on the primary tumor, but not on metastasis. Given the fact that the vast majority of cancer deaths results from formation of distant metastases, only the comprehensive preclinical studies addressing effects of exercise not only on the primary tumor, but also on metastatic dissemination are important [8]. Therefore, in the present study we investigated the effects of voluntary wheel running on formation of primary tumor and spontaneous lung metastasis in female Balb/C mice injected orthotopically with 4T1 breast cancer cells.

Materials and methods

Animals

Thirty-six female Balb/C mice were purchased from the Mossakowski Medical Research Centre (Warsaw), injected orthotopically with 1×10^4 of 4T1 breast cancer cells and randomly assigned to the 4T1 sedentary (4T1 sed; 20 mice) or 4T1 exercising group (4T1 ex; 16 mice). After injection, mice were placed in individual cages. In the case of the 4T1 ex group, the cages were equipped with wheels (Columbus Instruments, Columbus, OH, USA). The individual wheel count was recorded electronically every 10 s throughout the 5-week disease progression and the weekly covered distance was calculated based on the wheel count (the inner diameter of the wheel was 8.9 cm) using the following equation: $D=(N * 8.9 * \pi)/100$ [m]. These data was then used to calculate the mean total distance achieved by all mice and the mean velocity ($V=D/t$). The volume of the primary tumor was measured with calipers in the consecutive weeks of disease progression and calculated as described by [20]. Simultaneously, the body mass of each mouse was recorded. At the beginning of the 5th week of the disease, all animals were euthanized (ketamine and xylazine, 100 and 10 $mg \cdot kg^{-1}$, respectively). The spleens, primary tumors and lungs were excised and weighed. The lungs were subsequently fixed in formalin and pulmonary metastases were counted with the magnifying glass. Throughout the experiment, the animals were kept in a temperature-controlled environment (22-25°C), maintained on a 12-hour light/day cycle and given unlimited access to food (standard chow, AIN from Zoolab, Krakow, Poland) and water. One mouse from the 4T1 ex group was excluded from analysis since at the time of euthanization a primary tumor was not detectable. The experimental procedures involving animals were compliant with guidelines and were approved by the First Local Ethical Committee at the Jagiellonian University (Krakow, Poland, permit no: 140/2013).

Cell culture

The mouse mammary adenocarcinoma 4T1 cells were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI 1640 (IIET, Poland) with Opti-MEM® (Life Technologies, USA) (1:1 v/v) medium with 5% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc. UK), supplemented with 4.5 g/L glucose, 2 mM glutamine, 1.0 mM sodium pyruvate (all from Sigma-Aldrich, Germany) and antibiotics (penicillin and streptomycin-Polfa Tarchomin, Poland). Cell cultures

were maintained at 37°C in a humidified atmosphere with 5% CO_2. Prior to the transplantations, cells were trypsinized (IIET, Poland), centrifuged (200 g, 4°C, 5 min) and counted. 4T1 cells were resuspended in Hank's Balanced Salt Solution (HBSS; IIET, Poland) such that a suspension of 1×10^4 4T1 cells in 0.050 ml of HBSS was inoculated into the mammary fat pad of female Balb/C mice.

Nitric oxide spin trapping and EPR detection

Krebs-Hepes buffer (consisting of, in mM: NaCl 99; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.0; $CaCl_2$ 1.9; $NaHCO_3$ 25; glucose 11.1; and Na-Hepes 20) was filtered through a 0.22 μm paper syringe filter and equilibrated to pH 7.4. Following this step, the filtered buffer was deoxygenized by bubbling argon gas for at least 30 minutes. DETC (3.6 mg) and $FeSO_4 \cdot 7H_2O$ (2.25 mg) were separately dissolved under argon gas bubbling in two 10 ml volumes of ice-cold Krebs-Hepes buffer and kept under gas flow on ice until used. Freshly harvested thoracic aortas were cleaned of adherent fat, opened longitudinally, placed in 24-well plates filled with 100 μl Krebs-Hepes and preincubated for 30 minutes at 37°C. DETC and $FeSO_4 \cdot 7H_2O$ solutions were mixed 1:1 (v/v) to obtain 250 μl $Fe(DETC)_2$ colloid per well (final concentration 285 μM) and immediately added to the aortas in parallel with calcium ionophore A23187 (final concentration 1 μM) to stimulate eNOS and incubated at 37°C for 90 min. After incubation, each aorta was removed from the buffer, drained on a piece of kimwipe for 5 s and the wet mass was measured. Next, the aorta was frozen in liquid nitrogen into the middle of a column of Krebs-Hepes buffer. The needle-end was cut from a 1 ml insulin syringe, the plunger retracted 1 cm from the cut end, and 200 μl of the buffer was frozen in liquid nitrogen. Subsequently, the buffer was warmed for a few seconds to allow retraction and the aorta was placed on top and refrozen in liquid nitrogen. The syringe was then warmed once more to allow retraction of the column and an additional 200 μl of the buffer was pipetted on top of the frozen column, to allow freezing of the aorta in the middle of a 400 μl column of Krebs-Hepes buffer and was then stored in -80°C until measured. The frozen column was pushed out directly into a finger Dewar (Noxygen, Germany) containing liquid nitrogen. EPR spectra were obtained using an X-band EPR spectrometer (EMX Plus, Bruker, Germany), equipped with a rectangular resonator cavity H102. Instrument settings were: centre-field (B_0) 3276G, sweep 115G, microwave power 10 mW, modulation frequency 100 kHz, amplitude modulation 8 G, sweep time 60 s, and number of scans 4. Signals were quantified by measuring the total amplitude of the NO-DETC after correction of baseline as previously performed [21].

Measurement of blood and plasma parameters

Blood samples were collected from the right ventricle into the syringe containing heparin (10 U ml^{-1}). The blood count was performed using the animal blood counter Vet abc (Horiba Medical, France) and cytometric analysis of platelet activation was performed using a flow cytometer (LSRII and FACS/Diva ver. 6.0, respectively, Becton Dickinson, Oxford, UK). The rest of the blood was centrifuged at 1000 G/10 min/4°C to obtain plasma that was aliquoted to measure concentrations of 6-keto-PGF1α (Enzo Lifie Sciences, NY, USA) and P-selectin (Abcam, UK) with ELISA kits as well as NO_2^- and NO_3^- concentrations with an ENO-20 NOx Analyzer (Eicom Corp., Kyoto, Japan) as previously described [22].

Flow cytometry

Prepared "washed blood" was incubated with anti-mouse PE-conjugated GPIIb/IIIa or P-selectin antibodies for determination of their platelet surface expression and anti-mouse FITC-labeled anti-fibrinogen or von Willebrand (vWF) factor antibodies for determination of their membrane binding. Activation of circulating platelets was evaluated based on the measured expression/binding of surface membrane antigens. Platelets were identified by their forward- and side-scatter characteristics and were gated on the basis of the expression of platelet-specific antigen CD41/61. Isotype matched FITC- or PE-conjugated control antibodies were used to assess non-specific binding. Flow cytometric analyses of platelet activation was performed using flow cytometer software (LSRII and FACS/Diva ver. 6.0, respectively, Becton Dickinson, Oxford, UK). Measurements were made under the logarithmic gain and 10 000 events were collected. Appropriate color compensation was determined in samples singly stained with either FITC-conjugated anti-CD41/61 or PE-conjugated anti-CD41/61. Unstained platelets were used to establish the

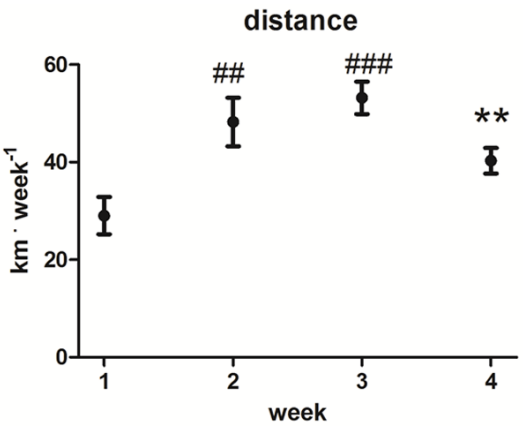

Figure 1. Mean distance covered in the consecutive weeks of breast cancer progression. Mice from the 4T1 ex group were placed individually in cages equipped with wheels and the wheel count was recorded continuously throughout the four weeks of the disease progression as described in *Materials and Methods*. The mean weekly distance in the 1st, 2nd, 3rd and 4th weeks of disease progression was calculated as described in *Materials and Methods*. At the beginning of the 5th week, the mice were euthanized. Statistical analysis was performed with two-sided Student T test and only $P<0.05$ were considered significant (n=15). The symbols # show statistical significance vs 1st week while the symbols *show statistical difference between the 3rd and the 4th week: ##/** ($P<0.01$), ### ($P<0.0001$). The data are presented as mean ± SEM.

level of autofluorescence set to fall within the first log order of brightness for each fluorescence channel. Suitable isotype controls were used as appropriate to set up the background noise at less than 1 percent. Events appearing above the background level were then recorded. Results were presented as the percentage of activation marker-positive events in platelets population.

Statistical analyses

Statistical analysis was performed in GraphPad Prism Software 5 (GraphPad Software, USA). The data were analyzed with two-sided T test or non-parametric Mann-Whitney test based on the results of D'Agostino and Pearson omnibus normality test. All data were presented as mean values ± SEM.

Results

Quantification of voluntary wheel running exercise along breast cancer progression

The mean total distance covered and the mean velocity achieved by 4T1 ex mice during four weeks of voluntary running were 170.45±47.5 km and 17.45±1.8 $m \cdot min^{-1}$, respectively. In the 1st week following cancer cells injection, the mice from the exercising group covered shorter distance than in the subsequent two weeks (**Figure 1**) (29±4 km in the 1st week vs 48±5 km, P=005, n=15 and 53±3 km, $P<0.0001$, n=15 in the 2nd and 3rd week, respectively). This effect was most likely due to progressive acclimatization of mice to the wheels. On the other hand, in the week prior to mice euthanization, the covered distance was lower than in the week before (40±2.6 km vs 53±3 km, P=0.0052, n=15) (**Figure 1**). This is likely to be attributed to the mice attaining advanced stages of the disease (**Figure 2A**).

Effects of voluntary activity on the progression of breast cancer

Primary tumors became palpable in all mice in the 3rd week of the disease and their mass increased afterwards (**Figure 2A**). There was no difference in the primary tumor volume between non-exercising 4T1 sed and exercising 4T1 ex mice except in the 3rd week when it was slightly higher in 4T1 sed mice (44.9±8 mm^3 for 4T1 sed mice vs 24.2±3.5 mm^3 for 4T1 ex mice, n=15-20, P=0.03). There was also no difference between the weight of primary tumors after their excision from 4T1 sed and 4T1 ex mice in the 5th week of the disease (**Figure 2B**). Interestingly, in the 5th week of the disease, the body weight of 4T1 ex mice tended to be slightly lower than the body weight of 4T1 sed mice (P=0.0525, n=15-20) (**Figure 2C**). Although the weight of the lungs was not different between 4T1 sed and 4T1 ex mice (**Figure 2D**), the number of pulmonary metastases was significantly higher in the 4T1 ex group as compared with the 4T1 sed group (1.6±0.6 in 4T1 sed vs 14.8±5.7 in 4T1 ex mice, P=0.035, n=9) (**Figure 2E**). Analysis of blood count revealed that the RBC number and the PLT number were lower in the 4T1 ex mice (**Table 1**). No difference between spleen weight, an indirect parameter of systemic inflammation [11], was found between the 4T1 sed and 4T1 ex mice.

Effects of voluntary activity on systemic endothelial function and NO bioavailability

In normal vascular physiology, endothelium-derived NO maintains the vascular wall in a quiescent state by inhibition of inflammation and cellular proliferation [23]. It was observed that an increased number of pulmonary metastases

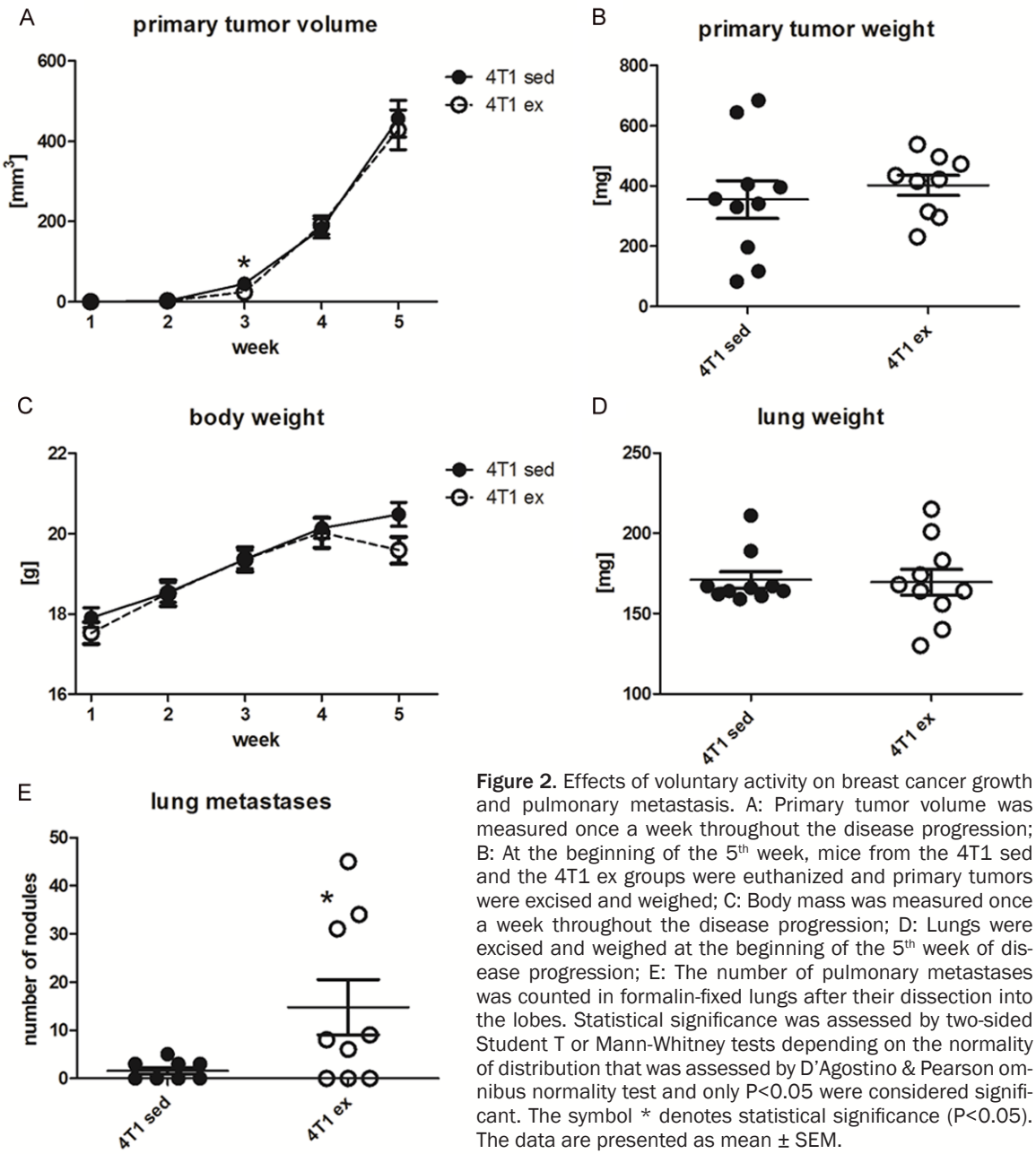

Figure 2. Effects of voluntary activity on breast cancer growth and pulmonary metastasis. A: Primary tumor volume was measured once a week throughout the disease progression; B: At the beginning of the 5th week, mice from the 4T1 sed and the 4T1 ex groups were euthanized and primary tumors were excised and weighed; C: Body mass was measured once a week throughout the disease progression; D: Lungs were excised and weighed at the beginning of the 5th week of disease progression; E: The number of pulmonary metastases was counted in formalin-fixed lungs after their dissection into the lobes. Statistical significance was assessed by two-sided Student T or Mann-Whitney tests depending on the normality of distribution that was assessed by D'Agostino & Pearson omnibus normality test and only $P<0.05$ were considered significant. The symbol * denotes statistical significance ($P<0.05$). The data are presented as mean ± SEM.

in 4T1 ex mice (**Figure 2E**) was associated with decreased production of NO in their aortas (45030±5636 AU for 4T1 ex vs 67860±4439 AU for 4T1 sed mice, P=0.0052, n=8-10) (**Figure 3A**) indicating more pronounced systemic endothelial dysfunction in exercising mice. On the other hand, the concentration of PGI_2 stable metabolite 6-ketoPGF1α was increased in the plasma of 4T1 ex mice (10112±1966 $pg \cdot ml^{-1}$ for 4T1 sed mice vs 20919±3144 $pg \cdot ml^{-1}$ for 4T1 ex mice, P=0.021, n=8) (**Figure 3B**) suggesting a compensatory activation of vascular PGI_2 production in the setting of compromised endothelial NO production [24], as reported also for other models [25]. Interestingly, the systemic NO bioavailability was not lower in 4T1 ex mice compared with 4T1 sed mice as evidenced by similar plasma concentrations of nitrite (NO_2^-) (**Figure 3C**) and nitrate (NO_3^-) (**Figure 3D**).

Effects of voluntary activity on platelet activation

It is known that platelets, once activated, shed P-selectin rapidly from their surface into the

Table 1. Blood count and spleen weight

	Experimental groups	
Parameter	4T1 sed	4T1 ex
RBC [M·µl^{-1}]	10.7±0.16 (n=10)	10.2±0.18 (n=7) (P=0.018)*
HGB [g·dl^{-1}]	14.6±0.9 (n=10)	16.2±0.3 (n=9)
WBC [K·µl^{-1}]	93.42±21.8 (n=10)	102.5±19 (n=9)
GRA [K·µl^{-1}]	61.5±14.6 (n=10)	72.1±0.5 (n=9)
LYM [K·µl^{-1}]	21.4±4.8 (n=10)	28±4.5 (n=9)
PLT [K·µl^{-1}]	855±34 (n=8)	609±82 (n=6) (P=0.013)*
Spleen weight [mg]	454±50.5 (n=11)	491±36 (n=9)

At the beginning of the 5th week of the disease, mice from 4T1 sed and 4T1 ex groups were euthanized. Blood count was performed and spleens were weighed. Statistical significance was assessed by two-sided Student T test or Mann-Whitney tests depending on the normality of distribution determined by D'Agostino & Pearson omnibus normality test and only P<0.05 were considered significant. The symbol * denotes statistical significance (P<0.05). Data are presented as mean ± SEM.

plasma [26]. Therefore, the plasma P-selectin concentration (**Figure 4A**) and the platelet activation *ex vivo* (**Figure 4B-E**) were measured to evidence that neither the plasma P-selectin concentration nor the platelet activation markers were increased in 4T1 ex compared with 4T1 sed mice.

Discussion

This study demonstrated that voluntary exercise increased formation of lung metastases in orthotopic syngeneic mouse model of breast cancer (**Figure 2E**). The pro-metastatic effects of voluntary exercise were associated with lower NO production by isolated aortas of exercising mice (4T1 ex) as compared with aortas isolated from sedentary control mice (4T1 sed) that were also injected with 4T1 cells (**Figure 3A**). However, no evident decrease in systemic NO bioavailability was identified (**Figure 3C** and **3D**) probably due to the compensation of impaired endothelial NO production by NO_3^-→ NO_2^-→NO reductive pathway as reviewed by [27]. Moreover, similar plasma concentrations of NO_2^- and NO_3^- in 4T1 sed and 4T1 ex mice in the terminal 5th week of disease progression indicated that there was no increase in systemic production of free radicals and oxidative stress in 4T1 ex mice (NO can undergo autoxidation to form NO_2^- and NO_3^- [28] at that stage of the disease. However, it cannot be excluded that at the beginning of the voluntary wheel running ROS and RNS associated with exercise were overproduced in breast cancer-bearing mice and exercise-induced oxidant stress could inhibit the signaling pathways responsible for genome stability [29] and, thus, promote breast cancer malignancy [30].

An increase of pulmonary metastasis in mice voluntarily running the wheels reported in this study is surprising, considering the strong epidemiological evidence that exercise not only reduces breast cancer risk [1, 31], but also exerts beneficial effects in patients with previously diagnosed mammary gland tumor [4-7]; though the studies showing no positive association between physical activity and breast cancer recurrence and mortality also exist [9, 10]. Similarly to human studies, the data derived from pre-clinical experiments with rodent models are also not consistent. In fact, while some reports show retardation of breast cancer progression in response to exercise [11-13, 18], others show no [14, 15, 32] or even the opposite effect [16].

An interesting study investigating effects of voluntary wheel running on the primary tumor growth and perfusion after orthotopic implantation of 4T1 breast cancer cells was conducted by [18]. The authors found that voluntary physical activity reduced primary tumor growth, improved perfusion of the tumor and increased apoptosis of breast cancer cells within the primary tumor. However, this study did not refer to the effects of exercise on the metastatic burden. In view of the fact that the majority of cancer deaths results from distant metastasis [8], the observation of increased spontaneous pulmonary metastases in mice injected orthotopically with 4T1 cells voluntarily running on the wheels (**Figure 2E**), despite no effects on the primary tumor (**Figure 2A** and **2B**), is even more alarming with respect to the potential hazard for patients with breast cancer undergoing voluntary exercise. It is also worth noting that these authors investigated effects of exercise on the classical vessels lined with endothelial cells in the primary tumor. Interestingly, it has been recently shown that primary tumors comprised of 4T1 cells are also capable of forming pseudovessels, a phenomenon known as vascular mimicry [33]. Such pseudovessels, usu-

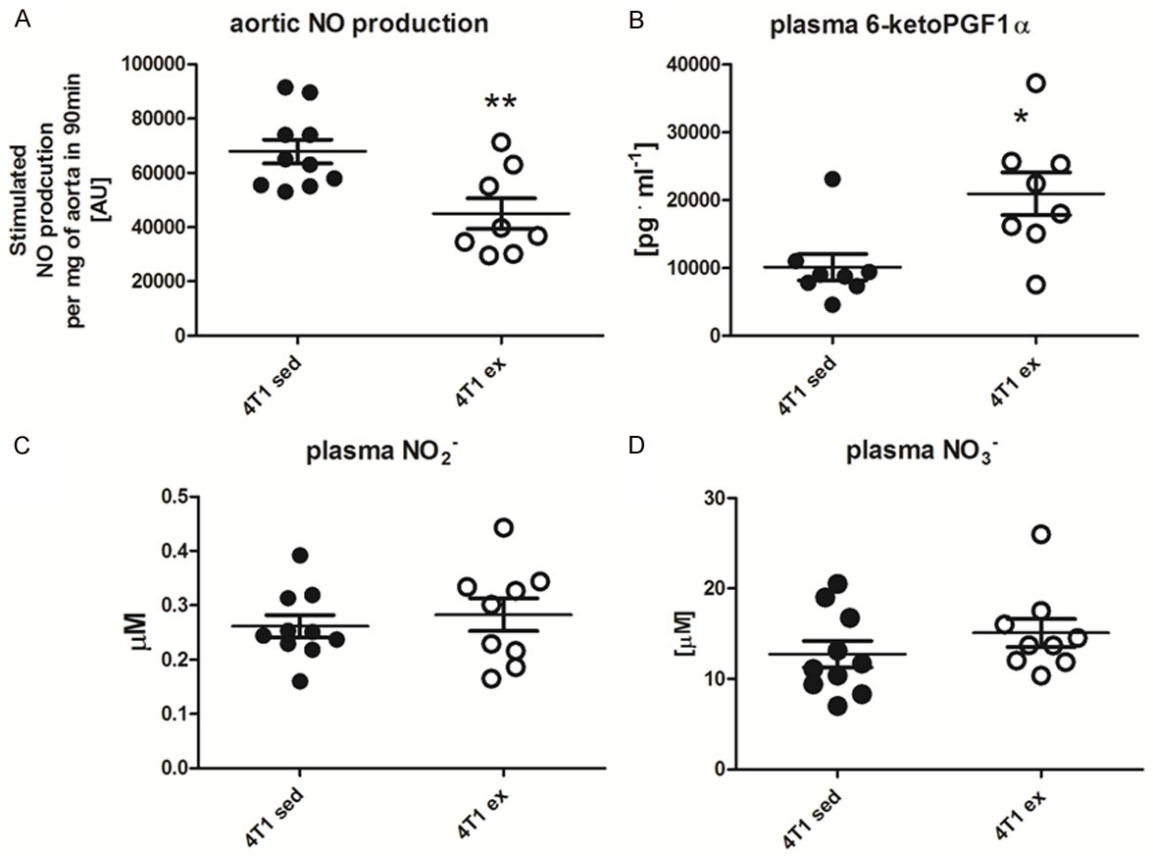

Figure 3. Systemic endothelial function and NO bioavailability. Aortas were excised, cleaned from the surrounding tissues. A: NO production by the aorta was measured as described in *Materials and Methods*; B: Plasma 6-ketoPGF1α production was measured by commercially available ELISA kit; C, D: Plasma NO_2^- and NO_3^- were determined as described in *Materials and Methods*. Statistical significance was assessed by two-sided Student T or Mann-Whitney tests depending on the normality of distribution assessed by D'Agostino & Pearson omnibus normality test and only $P<0.05$ were considered significant. The symbol * denotes statistical significance (*$P<0.05$, **$P<0.01$). The data are presented as mean ± SEM.

ally lined with tumor cells or a mixture of endothelial and tumor cells, converge with classical endothelium-lined vasculature and support circulation within the tumor thereby facilitating intravazation of cancer cells [34]. Moreover, [33] showed that improving perfusion in the pseudo-circulation within the primary tumor comprised of 4T1 cells by injection of exogenous anticoagulant warfarin promoted pulmonary metastases. Similar pro-metastatic effect in this model of breast cancer was also observed in the case of platelet depletion [35, 36]. These results suggest that improved perfusion of the primary tumor comprised of 4T1 cells caused by voluntary exercise may be responsible for increased metastatic spread in Balb/C mice. Obviously, further studies are needed to test such a hypothesis, but it is worth noting that the number of circulating platelets was significantly reduced in 4T1 ex mice (**Table 1**), concomitantly with increased number of metastatic foci in their lungs (**Figure 2E**).

As discussed above, regular exercise improves perfusion of primary tumor [18] and, thus, can facilitate intravazation of metastatic cancer cells. Tissue perfusion can also be increased by endogenous vasodilators such as prostacyclin (PGI_2) [37, 38], that is also a potent inhibitor of platelet activation [39, 40]. Indeed, the plasma concentration of PGI_2 stable metabolite 6-ketoPGF1α was higher in 4T1 ex mice (**Figure 3B**). Despite that fact, differences in basal platelet activation between 4T1 ex and 4T1 sed mice were not observed (**Figure 4**). However, lower number of circulating platelets in 4T1 ex mice (**Table 1**) suggested decreased probability of forming platelet aggregates in the circulation

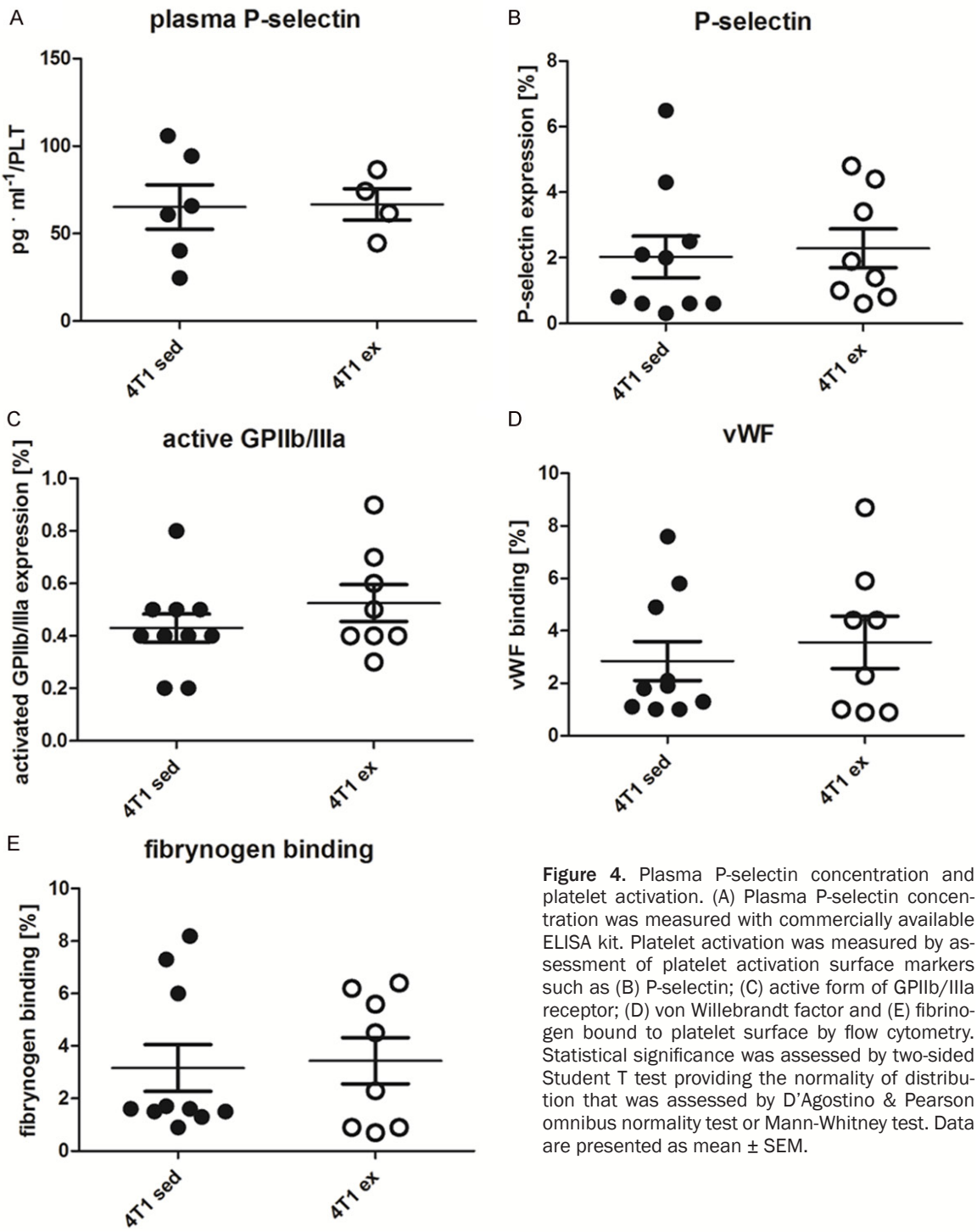

Figure 4. Plasma P-selectin concentration and platelet activation. (A) Plasma P-selectin concentration was measured with commercially available ELISA kit. Platelet activation was measured by assessment of platelet activation surface markers such as (B) P-selectin; (C) active form of GPIIb/IIIa receptor; (D) von Willebrandt factor and (E) fibrinogen bound to platelet surface by flow cytometry. Statistical significance was assessed by two-sided Student T test providing the normality of distribution that was assessed by D'Agostino & Pearson omnibus normality test or Mann-Whitney test. Data are presented as mean ± SEM.

of exercising mice in comparison with their sedentary counterparts. Last but not least, higher levels of circulating PGI_2 in exercising mice could also indicate the on-going angiogenesis [24], associated with increased endothelium permeability [41], which would facilitate extravasation of circulating cancer cells and crossing of the endothelial barrier during metastases [42].

It should be highlighted that in the present experiment we used voluntary wheel running as a model to study the effects of physical training on breast cancer progression. Such a model of

exercise is less stressful to animals than forced running on a treadmill or other forced exercise behaviors such as swimming. Thus, the exercise intervention imitates the self-paced voluntary exercise training sessions in humans, which have a number of health-related benefits. It should be stressed however, that the exercise intervention was applied to breast cancer-bearing animals with no history of training before the onset of illness. Our results clearly suggest that even voluntary exercise training intervention reported to improve health status elsewhere [2], when applied to breast cancer-bearing previously untrained animals, could stimulate disease progression. It might be argued that the differences between our results and the other findings presented in the literature concerning the effect of training on breast cancer progression in animals or patients [3, 4, 6, 7, 11-13, 18, 43] are due to increased oxidant stress of the untrained animals placed on the wheels. Interestingly, as discussed above, no evidence of higher systemic oxidative stress was found in 4T1 ex mice vs sedentary controls in the 5th terminal week of the disease (**Figure 3C** and **3D**). However, it cannot be excluded that at the beginning of wheel training, oxidative stress was more pronounced in yet untrained 4T1 ex mice and such unbalanced exercise-induced increase in ROS and RNS production in yet untrained individuals previously reported by [44], could facilitate effective metastases. Indeed, it is well documented that acute physical exercise increases ROS production in skeletal muscles [45]. Furthermore, it has recently been demonstrated that eight weeks of endurance training enhanced ROS production in non-phosphorylating (state 3) isolated rat skeletal mitochondria [46]. Therefore, increased number of pulmonary metastases in the previously untrained animals could well be due to the local training-induced enhancement of ROS and RNS production overreaching ROS and RNS defense mechanisms of cancer-bearing mice. However, further studies are needed to explain the role of the training-induced enhancement of ROS and RNS production on breast cancer progression in previously trained vs. untrained animals.

In summary, this study has demonstrated that spontaneous voluntary wheel running did not modify the volume and size of primary tumor, but promoted pulmonary metastasis. The effect was associated with decreased NO production in the isolated aorta, but not with activation of platelets suggesting that impaired endothelial function could contribute to that phenomenon. On the other hand, it might well be that exercise-induced improvement of tumor perfusion could also be involved, particularly in the case of aggressive breast cancer cells such as 4T1 cells forming pseudo-vessels [33]. Last but not least, it cannot be excluded that exercise-induced ROS and RNS production could contribute to that phenomenon. However, to elucidate the underlying mechanisms, further studies are warranted. Nevertheless, our results contradict the general belief that regular exercise always lowers breast cancer progression and call for revising the notion of the beneficial effects of exercise on cancer metastasis.

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Disclosure of conflict of interest

None.

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