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Vascular Modulation Through Exercise Improves Chemotherapy Efficacy in Ewing Sarcoma

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Abstract

Recent studies in mouse models of cancer have shown that exercise improves tumor vascular function, thereby improving chemotherapy delivery and efficacy. However, the mechanisms underlying this improvement remain unclear and the effect of exercise on Ewing sarcoma (ES), a pediatric bone and soft tissue cancer, is unknown. The effect of exercise on tumor vascular hyperpermeability, which inversely correlates with drug delivery to the tumor, has also not been evaluated. We hypothesized that exercise improves chemotherapy efficacy by enhancing its delivery through improving tumor vascular permeability. We treated ES-bearing mice with doxorubicin with or without moderate treadmill exercise. Exercise did not significantly alter ES tumor vessel morphology. However, compared with control mice, tumors of exercised mice had significantly reduced hyperpermeability, significantly decreased hypoxia, and higher doxorubicin penetration. Compared with doxorubicin alone, doxorubicin-plus-exercise inhibited tumor growth more efficiently. We evaluated endothelial cell Sphingosine-1-phosphate receptors 1 and 2 (S1PR1 and S1PR2) as potential mediators of the improved vascular permeability and increased function afforded by exercise. Relative to tumors from control mice, vessels in tumors from exercised mice had increased S1PR1 and decreased S1PR2 expression. Our results support a model in which exercise remodels ES vasculature to reduce vessel hyperpermeability, potentially via modulation

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

of S1PR1 and S1PR2, thereby improving doxorubicin delivery and inhibiting tumor growth more than doxorubicin alone. Our data suggest moderate aerobic exercise should be tested in clinical trials as a potentially useful adjuvant to standard chemotherapy for patients with ES.

Keywords

Ewing Sarcoma; angiogenesis; oncology; pediatric oncology; alternative medicine; exercise; chemotherapy delivery; S1PR

INTRODUCTION

Ewing sarcoma (ES), an aggressive bone and soft tissue sarcoma, is the second most common pediatric bone tumor and is treated with high-dose chemotherapy in combination with radiation and surgery. The need for high-dose chemotherapy is due in part to the low efficiency of drug delivery to the tumor because of poorly functional tumor vasculature, which is immature and hyperpermeable. On average, only 50% of ES tumor vessels are functional.^{1,2}

Inefficient tumor vessels create heterogeneous, disturbed blood flow that results in uneven chemotherapy delivery to the tumor, consequently reducing chemotherapy efficacy. Using mouse models of cancers primarily found in the adult population (melanoma, pancreatic, breast, and prostate cancer) we and others have demonstrated that exercise can remodel tumor vasculature and improve chemotherapy efficacy by increasing drug delivery³⁻⁸ as well as oxygenation. In prostate tumors, for example, increased tumor blood flow to the tumor after exercise decreased intratumoral hypoxia³⁻⁵ converting a hypoxic, aggressive tumor phenotype with poorly structured tumor vasculature to a more oxygenated, slower-growing tumor phenotype with organized, mature vasculature.^{3,5}

These studies suggest that exercise has a potential role as an adjuvant therapy to chemotherapy, yet the mechanisms by which exercise improves tumor vessel function remain unclear. One key aspect of tumor vascular remodeling for improved chemotherapy delivery is the reduction of hyperpermeability. Vessel hyperpermeability causes excessive fluid loss from the tumor vasculature to the interstitial space, resulting in impaired blood flow to the majority of the tumor and subsequent inadequate chemotherapy delivery.⁹ Tumor vascular leak is inversely correlated with chemotherapy delivery to the tumor as poorly functioning hyperpermeable tumor vessels cause leakage of drug at the periphery due to the high interstitial fluid pressure of a solid tumor.⁹

Vessel hyperpermeability is likely to be impacted by exercise via S1PR1 and S1PR2 on the surface of endothelial cells.^{10,11} These receptors act as shear stress responsive mechanoreceptors that regulate vessel barrier integrity through their signaling pathways.^{12,13} Shear stress is one mechanical stimuli exerted on endothelial cells by blood as it flows,¹⁴ and exercise creates laminar shear stress proportional to its intensity.¹⁵ S1PR1 expression suppresses hyperpermeability through the stabilization of vascular endothelial cadherin at adherens junctions in response to laminar flow.^{11,13} S1PR2 expression, which is associated with pathologic endothelium and increased vascular permeability, is upregulated by

disturbed blood flow, such as that found in tumors.^{12,16} Exercise has been demonstrated to increase S1P ligand expression in serum of human subjects,¹⁷ but regulation of the receptors in response to exercise has not been evaluated. We hypothesized that regulation of S1PR1 and S1PR2 correlates with tumor vascular remodeling in response to exercise-induced shear stress.

In this study, we used human ES xenografts as a pediatric cancer model in adolescent mice to show that a clinically relevant program of moderate intensity aerobic exercise improves chemotherapy efficacy against the tumor without increasing chemotherapy delivery to healthy organs. Further, we present the modulation of S1PRs as a potential molecular explanation for the improvement in vascular function attributed to exercise, in turn improving chemotherapy delivery and efficacy.

MATERIALS AND METHODS

Cell culture

A673 and TC71 human ES cells were acquired from the American Type Culture Collection. A673 and TC71 cells were cultured in DMEM (Caisson Labs) containing 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. TC71 cell media also contained 0.1 mM non-essential amino acids. Cells were passaged no more than 6 times prior to injection. A673 and TC71 cells were authenticated by short tandem repeat fingerprinting through the Characterized Cell Line Core at The University of Texas MD Anderson Cancer Center (Houston, TX; 3/31/017) and routinely tested for *Mycoplasma*.

Animals and experimental protocol

All animal experiments were approved by MD Anderson's Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards. Four- to six-week-old male nude mice were obtained from the Experimental Radiation Oncology Breeding Core at MD Anderson. All animal experiments were repeated at least three times.

A673 tumor cells (2.5×10^6) in 200 µl of phosphate-buffered saline (PBS) were injected subcutaneously into the backs of mice. When tumors were approximately 35–50 mm³ (7–10 days after injection), the mice were divided into 4 cohorts of 5–10 mice each with equal average tumor sizes: control (no exercise nor doxorubicin), exercise-only, doxorubicin-only, and doxorubicin-plus-exercise. Mice in the exercise-only and doxorubicin-plus-exercise groups were subjected to moderate intensity (60 – 70% VO₂ max¹⁸) treadmill running at 12 meters/minute for 45 minutes for 5 consecutive days per week for 2 weeks. Mice in the doxorubicin-only and doxorubicin-plus-exercise groups received intraperitoneal doxorubicin (2.5 mg/kg in 100 µl PBS) twice weekly for a total dose of 10 mg/kg. All mice were euthanized 21–25 days following tumor cell inoculation, 2 days after the final day of treatment (Fig. 1A). Tumors were harvested and fixed in formalin or frozen in optimal cutting temperature compound. The experiment was also conducted with TC71 cells (2×10^6).

For the assessment of doxorubicin delivery, 20 mice were injected with A673 cells (2.5×10^6). 10 tumor-bearing mice were treated with moderate-intensity treadmill running for 2

weeks (10 exercise sessions) and 10 served as controls. Seventy-two hours after the final exercise session, all exercised mice and 8 of the 10 control mice were injected with a bolus dose of doxorubicin. The remaining 2 mice served as negative controls for doxorubicin spectrophotometry and did not receive doxorubicin (Fig. 1B). The experiment was also conducted with TC71 cells (2×10^6).

Immunofluorescence staining

Paraffin-embedded slides were de-paraffinized using xylenes and ethanol and then antigen retrieval was performed in 20 $\mu\text{g}/\text{mL}$ proteinase K at 37°C for 40 minutes. Frozen sections were fixed in ice-cold acetone for 10 minutes. Slides were washed in PBS then incubated with the primary antibody diluted in Tween 0.3%/1% bovine serum albumin/5% normal goat serum overnight at 4°C. The primary antibodies used were rat anti-mouse CD31 at 1:50 (BD Pharmingen 553370), rabbit anti-mouse desmin at 1:50 (abcam15200), rabbit anti-mouse alpha-smooth muscle actin (α -SMA) at 1:100 (abcam5694), S1PR2 at 1:50 (Origene AP01198PU-N), and S1PR1 at 1:50 (Santa Cruz sc-25489 or Abcam Ab11424). AlexaFluor594- or AlexaFluor488-conjugated secondary antibodies (Invitrogen) were used at 1:1000 for 1 hour at room temperature. Nuclei were stained with Fluoro-Gel II with DAPI (Electron Microscopy Sciences). Images were captured with a Leica DM5500 B upright microscope imaging system (Leica Microsystems) and analyzed using SimplePCI6 (Legacy) or Adobe Photoshop software.

For the quantification of tumor vasculature, the areas of CD31-positive structures (microvessel density) were measured and the numbers of visible lumens, vessels, and vessels with lengths $> 100 \mu\text{m}$ were counted in 5 random 10 \times magnification photographs of each slide. For dextran and S1PR analyses, the number of dextran- or S1PR-positive vessels were compared with the number of CD31-positive vessels. For desmin and α -SMA analysis, areas positive for either desmin or α -SMA were measured in 5 sections and averaged to obtain one value for each tumor. The individual averages for all tumors within a treatment group were then averaged to determine the group average and standard error of the mean (SEM).

Vessel function quantification

Mice received an intravenous injection of 1 mg of high molecular weight (2×10^6 kDa) dextran–fluorescein isothiocyanate (FITC; Sigma-Aldrich) into the lateral tail vein immediately prior to euthanasia. Dextran-FITC was visualized by fluorescence microscopy.

To quantify vessel leak represented by dextran staining, we averaged the area of dextran immunofluorescence in 5 random 10 \times or 20 \times magnification photographs of each slide to obtain one value for each tumor. Individual averages for all tumors within a treatment group were then averaged to determine the group average and SEM. The dextran-positive area was then compared against the CD31-positive area to identify vessel leaks.

qPCR

Tumor samples (~30 mg) frozen in OCT compound from ES tumor-bearing mice were thawed in warm PBS. The samples were transferred to 595 μl of RLT lysis buffer (Qiagen) plus 6 μl β -mercaptoethanol (Sigma) and homogenized. The homogenate was centrifuged

for 3 min at 10,000×g. RNA was isolated from the supernatant using the Rneasy Mini Kit (Qiagen). Frozen organ samples (~30 mg) from TC71 tumor-bearing mice were homogenized in 1 mL TRIZOL reagent and RNA was isolated per manufacturer instructions.

cDNA was generated from 0.5 µg of each RNA sample using the Omniscript Reverse Transcription Kit (Qiagen). Single reactions were prepared for each sample with 1 µl cDNA in a final volume of 10 µl using iQ SYBR® Green Supermix (Bio-Rad). Carbonic anhydrase IX (CAIX; F:CACTCCTGCCCTCTGACTTC, R:TCTCATCTGCACAAGGAACG), hypoxia-inducible factor-1alpha (HIF1α; F:TTCACCTGAGCCTAATAGTCC, R:CAAGTCTAAATCTGTGTCTG), S1PR1 (F:TTGAGCGAGGCTGCTGTTT, R:TAGAGGGCGAGGTTGAGTGAG), and S1PR2 (F:GTCAGCATTCTGTCTGTCTATC, R:CAAGCCAGTCAAGGCTATGT) primers, as well as internal control primers 18S rRNA (F:GGGAGGTAGTGACGAAAATAACAAT, R:TTGCCCTCCAATGGATCCT) and GAPDH (F:AACAGCAACTCCCACTCTTC, R:CCTGTTGCTGTAGCCGTATT) were synthesized by Integrated DNA Technologies. Quantitative PCR was run on a LightCycler® 480 Instrument II (Roche). The cycling conditions were: 95°C for 10 min; 40 cycles of 95°C/30 s, 53°C/1 min for hypoxia samples or 50°C/1 min for S1PR samples; 95°C/1 min; 95°C/10 s, 65°C/1 min, 97°C/continuous. The baseline adjustment method of the LightCycler® 480 Instrument II (Roche) software was used to determine the Ct in each reaction. A melting curve was constructed for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimer. All samples were amplified in triplicates, and the mean mRNA was used for further analysis.

Doxorubicin quantification

Mice in the control and exercise-only groups (Fig. 1A) were injected with 10 mg/kg doxorubicin via the tail vein 72 hours after the last exercise session (Fig. 1B). Thirty minutes later mice were euthanized, and tissues were immediately harvested, weighed, and homogenized in acid isopropanol/Triton-X as described previously.¹⁹ Doxorubicin fluorescence was read using a spectrophotometer ($\lambda = 470$) and compared to a standard curve. Values were then normalized against the weight of tissue input and the background reading of a tumor from a control mouse that received no doxorubicin.

Statistical analysis

All values are reported as means \pm SEMs. *In vitro* and *in vivo* data are representative of at least 3 individual experiments. The tumor vasculatures of the different treatment groups were compared using an unpaired parametric t-test assuming a Gaussian distribution. P-values < 0.05 were considered significant. These statistical analyses were performed with GraphPad Prism6.

Since tumor growth is non-linear and tumor size is not normally distributed, square root transformation is applied for tumor volume analyses. Linear mixed model was used to evaluate the effect of treatment on square root transformed tumor size. Individual intercepts were considered as random effects, treatment and time (Day) were considered as fixed effects. Interaction between treatment and time was also considered in the model. The model

is as follows: Model : $\sqrt{\text{Tumor size}} = \text{Treatment} + \text{Time} + \text{Treatment} * \text{Time}$, with random intercept for each individual. Tumor volume computations were carried out in RStudio version 1.0.136.

RESULTS

Exercise does not significantly alter vessel morphology

To investigate whether moderate treadmill exercise morphologically changes ES tumor vasculature, we first compared the tumor vessels from tumor-bearing exercised or non-exercised mice (Fig. 2A and 3A). Neither A673 nor TC71 tumors of exercised or control mice differed significantly in open lumens, total vessels, microvessel densities or numbers of elongated vessels (Fig. 2B and 3B).

Pericytes are associated with vascular remodeling and function.²⁰ To investigate whether exercise changed their prevalence we evaluated desmin and α -SMA in tumors of exercised or non-exercised mice. Both A673 and TC71 tumors from exercised mice had significantly greater desmin-positive vessels than tumors from the control mice ($p = 0.0007$; Fig. 2C and $p = 0.0198$; Fig. 3C). The number of α -SMA-positive vessels was also significantly greater in A673 tumors (Fig. 2D) and approached significance in TC71 tumors ($p = 0.0891$; Fig. 3D) of exercised compared to control mice.

Exercise reduces tumor vessel hyperpermeability

Aside from the increase in mural cell coverage, we did not observe the striking changes in tumor blood vessel phenotype that have been reported in other tumor types.^{3,6} Therefore, we considered another important regulator of chemotherapy delivery to solid tumors: vascular leak. High molecular weight dextran was used to determine whether exercise reduced the hyperpermeability of ES vessels. The proportion of hyperpermeable vessels in both A673 and TC71 tumors from exercised mice (43.89% and 48%, respectively) was significantly lower than that in tumors from non-exercised mice (76.77%, $p = 0.0237$; 147.5%, $p = 0.0673$, respectively; Figs. 4A and B).

Critical mediators of blood vessel permeability are S1PR1 and S1PR2, expressed on the surface of endothelial cells and pericytes. Compared with A673 tumors from non-exercised mice, those from exercised mice had 16% higher S1PR1 expression ($p = 0.04$; Figs. 4C and D) and 50.6% lower S1PR2 expression ($p = 0.12$; Figs. 4E and F). S1PR1 and S1PR2 expression did not change after exercise in lungs (Fig. 4G) or gastrocnemius muscles (Fig. 4H).

Exercise improves tumor hypoxia and chemotherapy delivery and efficacy

We hypothesized that improved vessel permeability would allow for blood flow to a greater area of the tumor. Thus, we evaluated tumor hypoxia as a surrogate for blood delivery. The mRNA levels of HIF-1 α were 41.28% lower ($p = 0.26$; Fig. 5A) and those of CAIX were significantly lower by 51.23% ($p = 0.03$; Fig. 5B) in A673 tumors from exercised mice relative to tumors from non-exercised mice.

Improved blood flow in tumors of exercised mice compared to non-exercised mice suggested that exercise could increase delivery of chemotherapy to the tumor. Both A673 and TC71 tumors from exercised mice had significantly higher doxorubicin levels than tumors of non-exercised mice after a single dose of the drug ($p = 0.0365$, Fig. 5C; $p = 0.0058$, Fig. 5E). Importantly, doxorubicin levels in non-tumor-bearing organs (heart, liver, muscle, and lungs) of exercised and non-exercised mice did not differ significantly (Figs. 5D and F).

To further investigate the effect of exercise on chemotherapy delivery to ES, we used moderate intensity daily treadmill running and treated tumor-bearing mice with doxorubicin, exercise, or doxorubicin combined with exercise (Fig. 1A). Experiments resulted similarly each time; pooled data is shown in figures 5G and H. In a single representative experiment of A673 or TC71, mice treated with doxorubicin+exercise had smaller tumors than those treated with doxorubicin alone (43.9%, n.s. or 58.4%, $p = 0.04$, respectively). Tumors from mice treated with the combination of exercise and doxorubicin were significantly smaller than tumors from mice treated with doxorubicin alone in both the A673 and TC71 models ($p = 0.02$ and $p < 0.0001$, respectively; Figs. 5G and H).

DISCUSSION

Our study uniquely demonstrates that exercise reduces tumor vascular permeability and improves oxygenation in a mouse model of pediatric cancer. Exercise improved doxorubicin delivery thus enhancing its efficacy and resulting in greater inhibition of ES growth but did not increase chemotherapy delivery to non-tumor bearing sites. This study also suggests that modulation of S1PR1 and S1PR2 expression by exercise may be partially responsible for improved tumor vascular function.

The tumor vascular response to exercise appears to be partially dependent on tumor type. Others have demonstrated that exercise changes microvessel density, vessel length, or vessel patency; these were unchanged in our model.^{3,5,6,8} We did, however, have a similar observation in an increase in pericyte coverage.^{3,6,8} ES tumor vasculature is unique in that ES tumor cells are able to directly line vascular spaces and create blood lakes.²¹ Further, unlike pancreatic tumors which have compressed vessels or melanoma tumors which have very few elongated vessels with open lumens, ES tumor vessels have visible lumens even in tumors of untreated mice. Thus, the impact of exercise on tumor vessel morphology may be less substantial.

In the present study, we demonstrated that exercise additionally reduces excessive tumor vascular leak. This is important because with reduced vessel hyperpermeability chemotherapy can more efficiently reach the tumor, as permeability is inversely correlated with drug delivery to tumors.²² A reduction in vessel permeability would allow for greater delivery of not only chemotherapy but also blood, providing a mechanism for the work of others who have demonstrated that exercise increases blood perfusion and oxygenation of tumors.^{3,5,8}

In our mouse models of ES, we also found reduced tumor hypoxia after exercise in one tumor model, A673, but not the other, TC71. We suspect the lack of difference in the TC71 model is due to the extensive necrosis noted in tumors of all four treatment groups. Because tumor vasculature is dysfunctional, tumor blood perfusion is heterogeneous, resulting in tissue hypoxia.²³ The reduction of intratumoral hypoxia through exercise has been demonstrated in mouse models of prostate⁴⁻⁵ and breast cancers.³ Tumor hypoxia contributes to a more aggressive tumor phenotype²⁴⁻²⁵ and greater metastatic potential²⁶⁻²⁹ as well as chemotherapy and radiation resistance.³⁰⁻³¹ Thus, hypoxia reduction after exercise may have positive implications for patient outcomes.

Similar to our own findings, exercise has been found to increase drug delivery to the tumor.³⁻⁸ In the present study, we found that exercise does not, however, increase chemotherapy delivery to non-tumor bearing organs, including the heart, lungs, liver, and skeletal muscle. The clinical implications of this data are relevant to pediatric cancer survivors since survivors often die at younger ages than their peers owing to treatment-related late effects, such as heart failure or pulmonary disease.³²

Shear stress has been proposed to mediate exercise-induced improvements in tumor vascular function.³⁻⁶ S1PR1 and S1PR2 regulate endothelium in vascular disease^{10,33-34} and cancer³⁵⁻³⁸ and are shear stress responsive.¹²⁻¹³ S1PR1 signaling promotes tight intercellular junction assembly to appropriately modulate vascular permeability^{13,38} and induces vessel maturation.³⁷ Conversely, S1PR2 signaling increases vascular permeability^{10,39} and is inhibited by laminar shear stress.¹² Here, we show that S1PR1 and S1PR2 on tumor vasculature can be exploited with exercise, as exercise increased S1PR1 and decreased S1PR2. Consistent with increased doxorubicin delivery to tumors but not other organs, exercise did not impact S1PR1 and S1PR2 expression in lungs (where S1PR expression is prevalent^{40,41}) or gastrocnemius muscle.

ES patients are likely to benefit from improved tumor vascular function as a means to increase chemotherapy delivery. Prior studies have incorporated anti-angiogenic drugs into treatment regimens to improve tumor vessel function and induce vascular remodeling in combination with chemotherapy and radiation. Using this approach, one study increased the 24-month event-free survival of patients with ES and isolated pulmonary metastases compared with historical controls but reported adverse events including acute respiratory distress syndrome and pulmonary hemorrhage.⁴² Exercise may be one method to improve tumor vasculature while circumventing the undesired side-effects of standard therapies or eventual drug resistance. Clinical trials are needed to determine whether exercise has the same impact in patients as it does in mice.

Future clinical trials may include biomarkers to assess the vascular response to exercise. Previously we demonstrated that Thrombospondin-1 (TSP-1) is a key anti-angiogenic protein in the normalization of vasculature induced by exercise via calcineurin-NFAT-TSP-1 signaling.⁶ S1PR1 has been shown to induce calcium mobilization, enhancing NFAT-TSP-1 activity.⁴³ TSP-1 may serve as a potential serum biomarker for exercise-induced vascular remodeling, and future work will aim to elucidate the relationship between S1PR1 and TSP-1 in response to exercise.

The impact of the immune system was not accounted for in our mouse models that utilized nude, male mice. ES xenografts cannot grow in immunocompetent mice and there are no transgenic models of ES. However, further work is warranted in an immunocompetent model since the immune system is implicated in exercise induced changes in growth and sex hormones⁴⁴ which may impact tumor growth. This work is also limited by using subcutaneous models. Although subcutaneous tumor models have proven to be useful for evaluating molecular mediators of tumor vasculature^{45,46,47} and novel therapies for the treatment of ES,^{48,49,50} the findings reported here should be strengthened using orthotopic ES tumors. Additionally, our studies provided a limited number of days of exercise in a non-variable fashion with controlled settings on a treadmill. This is not unique to our study³⁻⁸ since it is necessary to demonstrate that the effects of exercise can be replicated. Exercise effects may be even more robust in prolonged doses of chronic exercise.

In the present study, we determined that moderate aerobic exercise remodeled ES tumor vasculature and modulated the S1PR1 and S1PR2 balance, thereby improving chemotherapy delivery and inhibiting tumor burden more than chemotherapy alone in mice. Notably, moderate aerobic exercise is feasible in adult and pediatric cancer patients undergoing treatment.⁵¹⁻⁵³ Our pre-clinical data suggests that clinical trials testing moderate aerobic exercise as a potential method to improve chemotherapy delivery and efficacy against ES should be performed.

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Abbreviation

S1PR	sphingosine-1-phosphate receptor
ES	Ewing sarcoma
NFAT	nuclear factor of T cells
Tsp-1	thrombospondin-1
PBS	phosphate buffered saline
Fig	figure
α-SMA	α-smooth muscle actin
SEM	standard error of the mean

CAIX	carbonic anhydrase IX
HIF1α	hypoxia-inducible factor-1alpha

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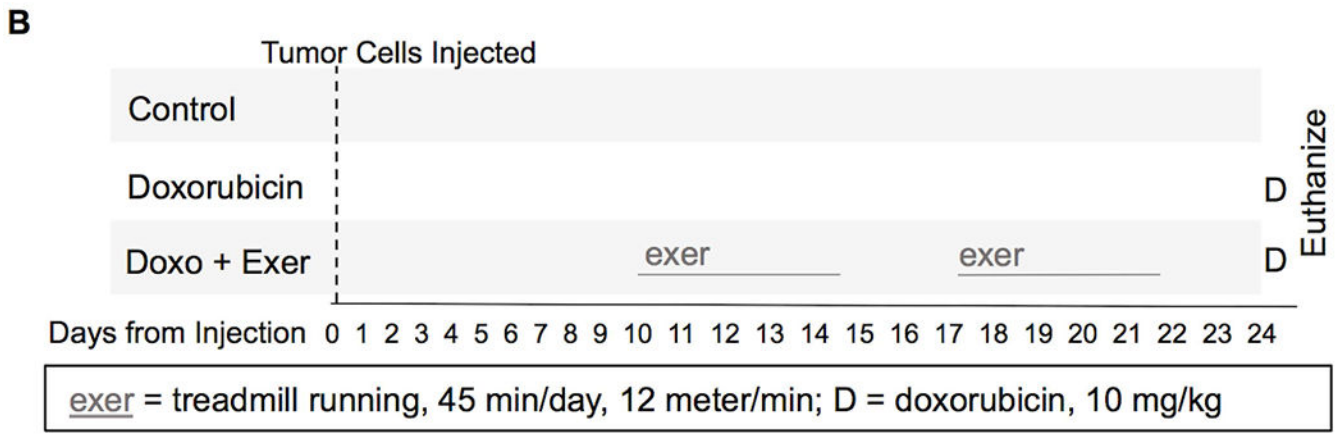
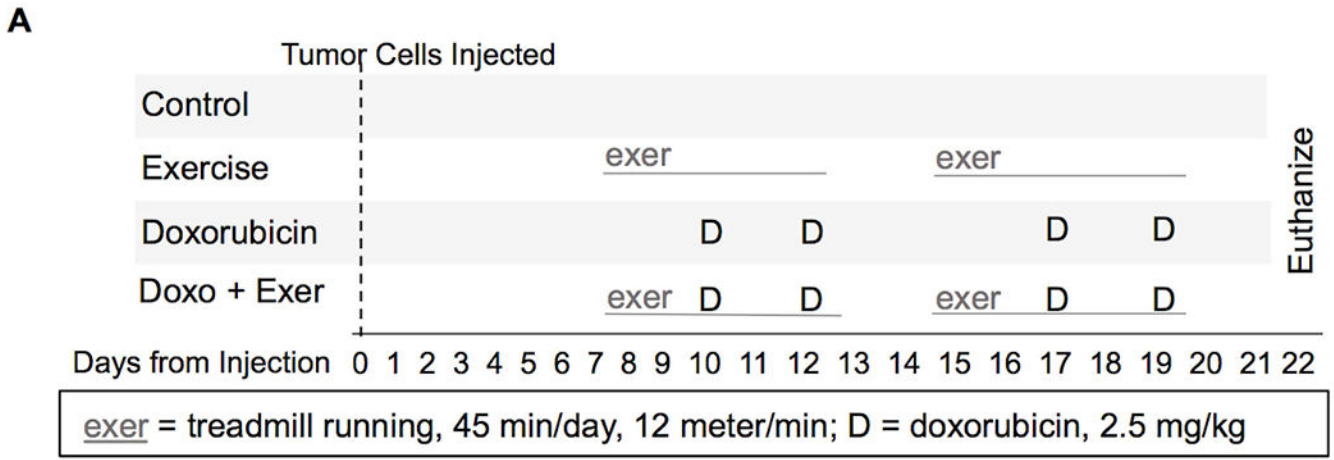


Figure 1. Experimental Schemas. (A) Ewing sarcoma tumor-bearing mice were divided into 4 groups: control, exercise, doxorubicin, or doxo + exer. Doxorubicin was administered twice/week for 2 weeks and exercise was performed 5 days/week for 2 weeks. (B) Tumor-bearing mice were divided into 3 groups: control, 1 dose doxorubicin, or 1 dose doxorubicin after 10 days of exercise. 72 hours after the final exercise, doxorubicin and doxo + exer mice received a bolus dose of doxorubicin followed by euthanasia. Doxorubicin was quantified by spectrophotometry and normalized against tumors or organs from control mice that did not receive doxorubicin or exercise.

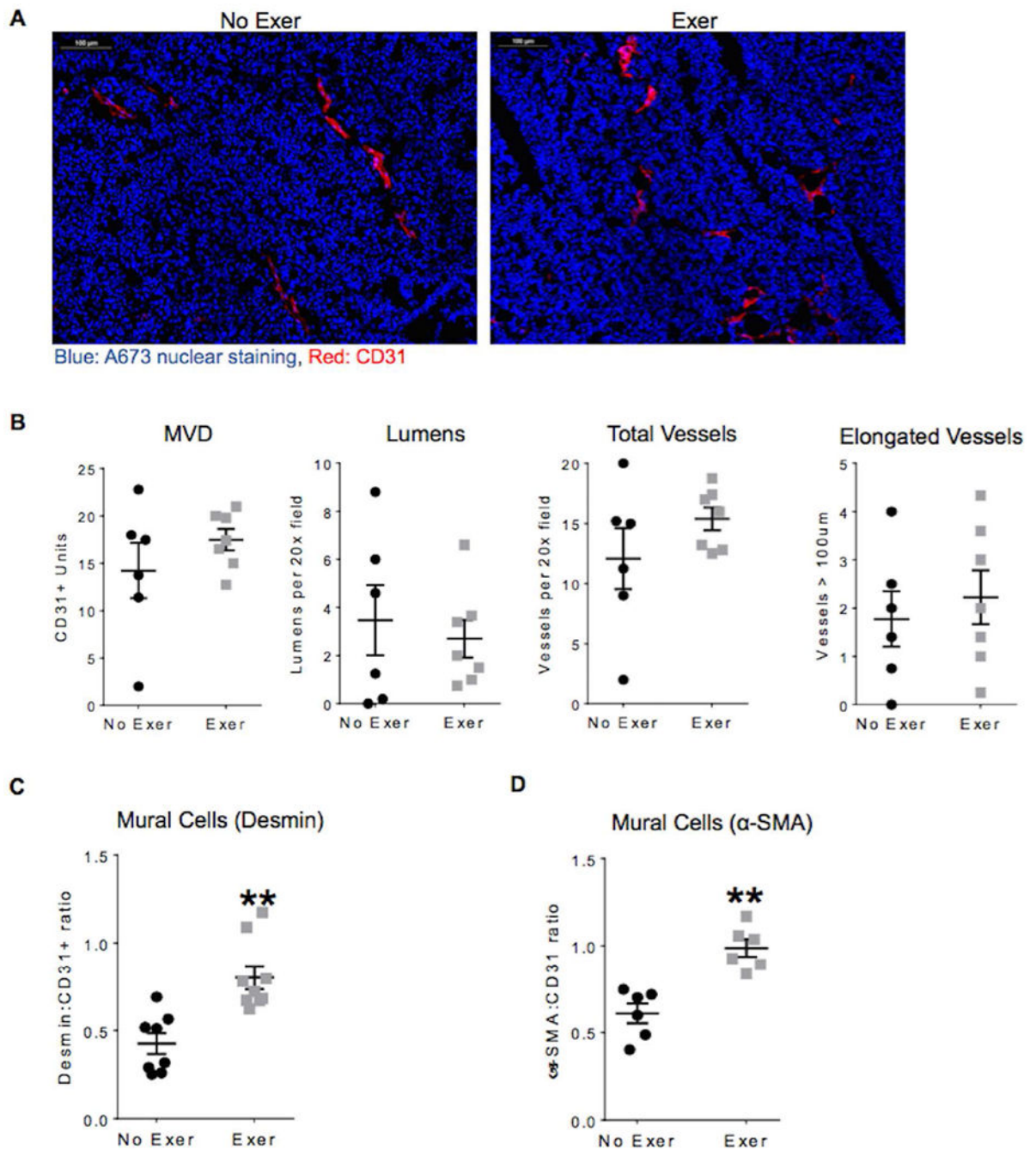


Figure 2.

Exercise does not significantly alter Ewing Sarcoma vessel morphology but increases mural cell density in A673 tumors. After A673 tumors were measurable, mice were randomized into No Exercise or treadmill Exercise groups. Tumors were harvested 21 days later. (A) Representative images of CD31 immunofluorescence (red) with DAPI staining to visualize nuclei (blue) in A673 tumors from non-exercised mice and exercised mice. (B) Microvessel densities (MVD), numbers of visible lumens, total numbers of vessels, and numbers of vessels > 100 μ m (elongated) were counted in 5 random sections/tumor and averaged to

obtain a value per tumor in non-exercised (black circles) and exercised (gray squares) mice. Bars represent means \pm SEM, $n = 6-7$. Mural cell density was also measured on 5 random sections/tumor and averaged. (C) Mean Desmin:CD31 ratio \pm SEM for individual A673 tumors, $n = 8-9$, $**p = 0.00007$. (D) Mean α -SMA:CD31 ratio \pm SEM for individual A673 tumors, $n = 6$, $**p = 0.0006$.

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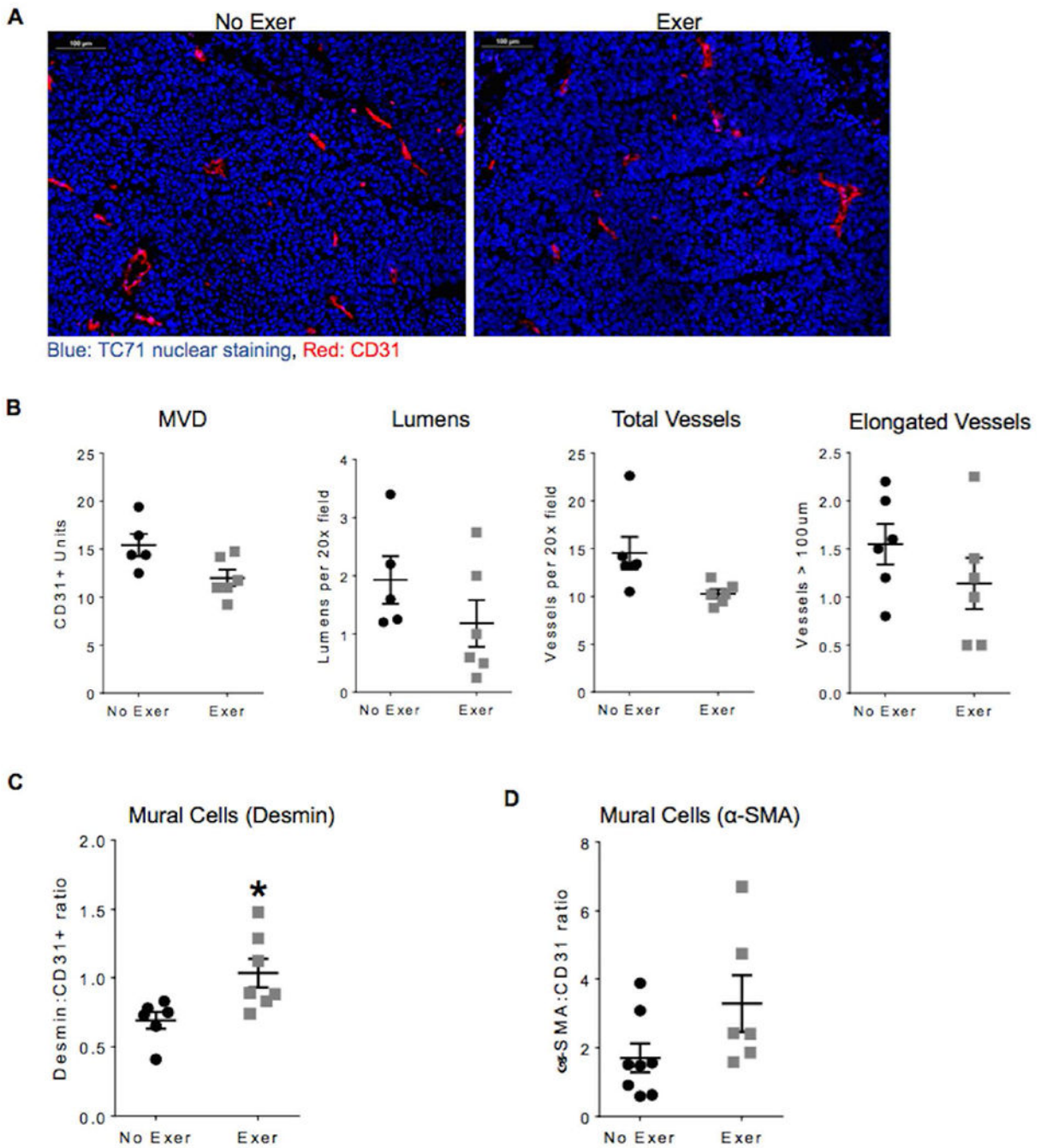


Figure 3. Exercise does not significantly alter Ewing Sarcoma vessel morphology but increases mural cell density in TC71 tumors. After TC71 tumors were measurable, mice were randomized into No Exercise or treadmill Exercise groups. Tumors were harvested approximately 21 days later. (A) Representative images of CD31 immunofluorescence (red) with DAPI staining to visualize nuclei (blue) in TC71 tumors from non-exercised mice and exercised mice. (B) Microvessel densities (MVD), numbers of visible lumens, total numbers of vessels, and numbers of vessels > 100 µm (elongated) were counted in 5 random sections/

tumor and averaged to obtain a value per tumor in non-exercised (black circles) and exercised (gray squares) mice. Bars represent means \pm SEM, $n = 5-6$. Mural cell density was also measured on 5 random sections/tumor and averaged. (C) Desmin:CD31 ratio \pm SEM for individual tumors, $n = 6-7$, $*p = 0.0198$. (D) α -SMA:CD31 ratio \pm SEM for individual tumors, $n = 6-8$, $p = 0.0891$.

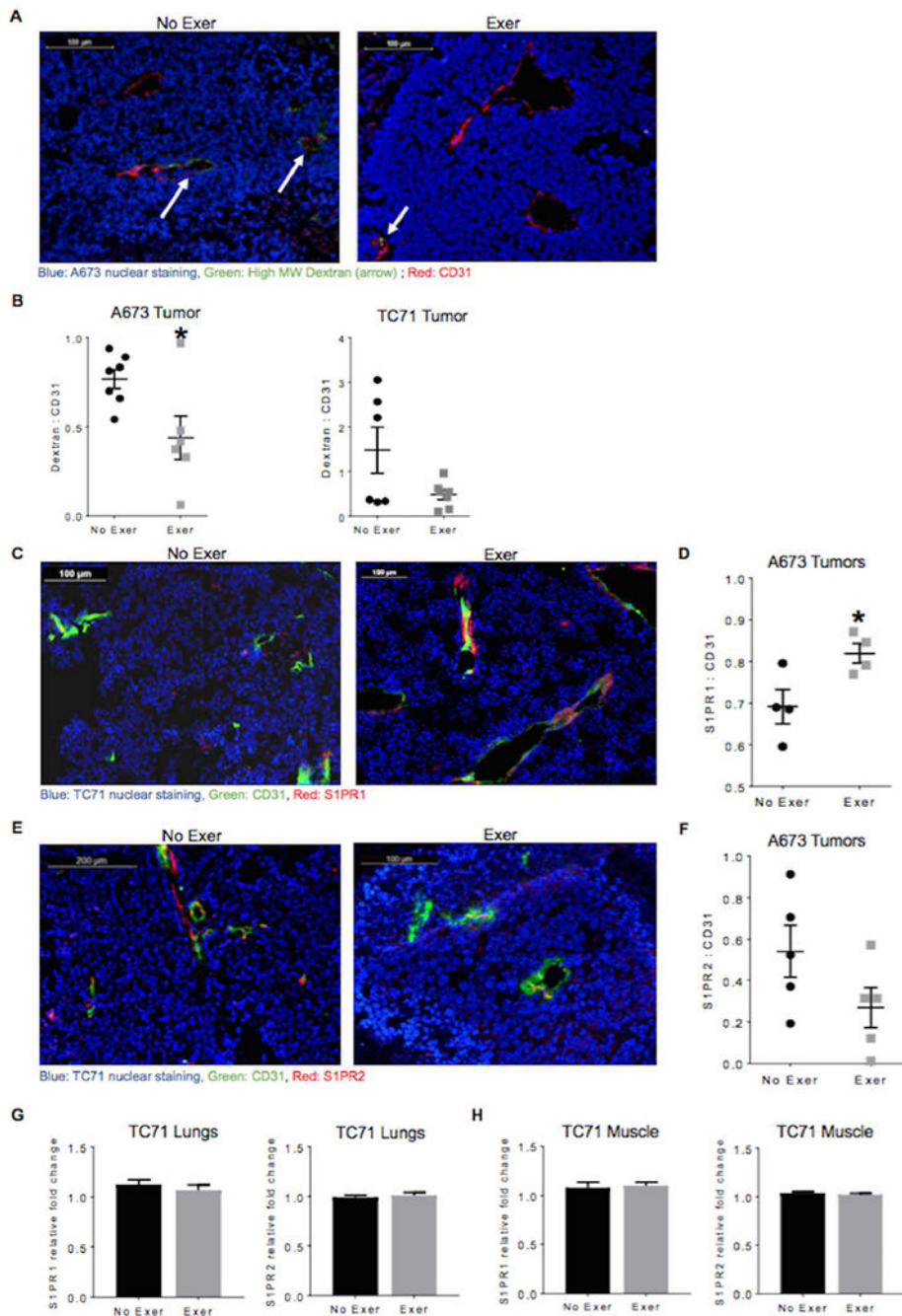


Figure 4. Exercise reduces tumor vessel permeability, increases tumor S1PR1, and decreases tumor S1PR2. Ewing sarcoma tumor bearing mice were divided into 4 groups: control, exercise, doxorubicin, or doxo + exer. Tumors were harvested approximately 21 days later. Immediately prior to euthanasia, mice in each group were injected with High Molecular Weight (2,000 kDa) dextran-FITC. (A) Representative images of dextran-FITC (green) and CD31 (red) immunofluorescence in A673 tumor vasculature of non-exercised mice compared to exercised mice. Arrow indicates a dextran positive vessel. (B) Mean

dextran:CD31 ratio \pm SEM for individual A673 tumors, $n = 6-7$, $*p = 0.02$ and TC71 tumors, $n = 6-7$, $p = 0.067$. (C) Representative images of CD31 (green) and S1PR1 (red) immunofluorescence with DAPI staining to visualize nuclei (blue) in TC71 tumors. (D) Mean S1PR1:CD31 ratio of immunofluorescence \pm SEM for individual A673 tumors, $n = 4-6$, $*p = 0.04$. (E) Representative images of CD31 (green) and S1PR2 (red) immunofluorescence on TC71 tumors with DAPI staining to visualize nuclei (blue). (F) Mean S1PR2:CD31 ratio of immunofluorescence \pm SEM for individual A673 tumors, $n = 4-6$. (G) mRNA levels of S1PR1 and S1PR2 in lungs and (H) muscle in TC71 tumor-bearing mice relative fold change compared to GAPDH housekeeping gene in tumor homogenates assessed by qPCR, mean \pm SEM for individual tumors, $n = 7-8$.

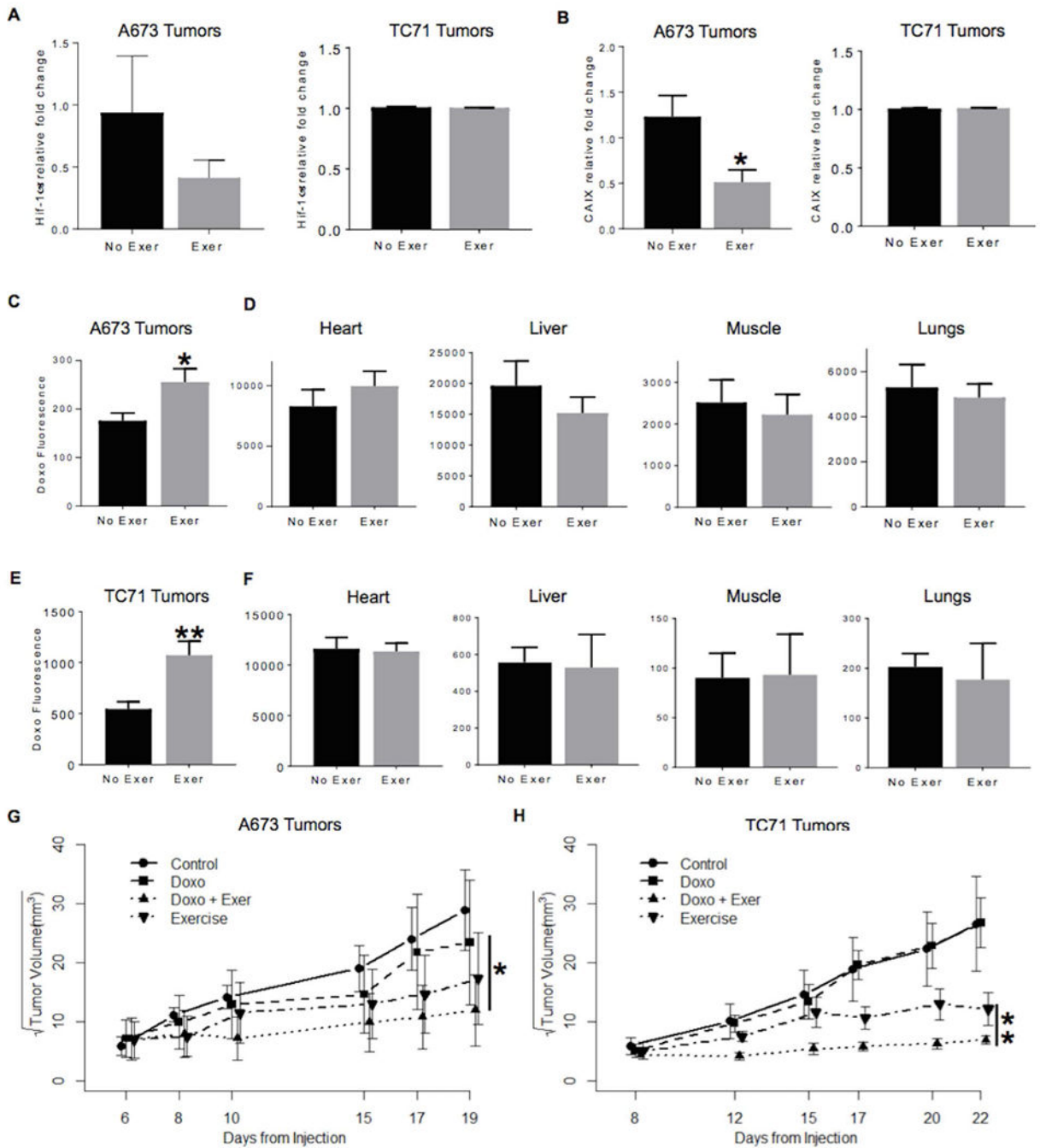


Figure 5. Exercise reduces hypoxia, increases doxorubicin delivery specifically to the tumor, and improves chemotherapy efficacy. (A, B) mRNA levels of Hif-1α and CAIX relative fold change compared to 18S housekeeping gene in tumor homogenates as assessed by qPCR, mean ± SEM for individual tumors, $n = 6-8$, $*p = 0.03$. (C) Spectrophotometry quantification of doxorubicin in A673 tumors harvested from mice after two weeks of exercise, mean ± SEM for individual tumors, $n = 7-9$, $*p = 0.0365$, and of (D) non-tumor-bearing organs of non-exercised and exercised mice with A673 tumors; mean ± SEM, $n =$

7-9. (E) Spectrophotometry quantification of doxorubicin in TC71 tumors harvested from mice after two weeks of exercise, mean \pm SEM for individual tumors, $n = 8-10$, $**p = 0.0058$, and of (F) non-tumor-bearing organs of non-exercised and exercised mice with TC71 tumors; mean \pm SEM, $n = 8-10$. (G, H) Volumes of A673 ($n = 5-7$) and TC71 ($n = 4-5$) tumors were measured on indicated days, mean \pm SEM, $*p = 0.02$ and $*p = <0.0001$, respectively.

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