

Effects of treadmill training on combined goserelin acetate and doxorubicin-induced osteopenia in female rats

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Abstract

Objectives: This study examined individual and combined effects of the cancer treatments goserelin acetate (GA) and doxorubicin (DOX) on bone and determined if treadmill running (TM) provides osteoprotection. **Methods:** Ten-week-old female Sprague-Dawley rats were randomly assigned to sedentary (SED) or TM groups. SED received GA, DOX, combined GA and DOX (GA+DOX), or placebo and maintained normal cage activity. TM received GA, DOX, GA+DOX, or placebo and participated in a progressive motorized treadmill protocol. After 8 weeks, tibiae were evaluated using micro computed tomography. **Results:** Negative drug effects were observed in cancellous bone (bone volume/tissue volume, trabecular number, trabecular thickness, trabecular spacing; $P<0.05$). An additive bone volume/tissue volume and trabecular spacing effect was observed in SED GA+DOX (vs. SED+GA and SED+DOX, $P<0.05$) but not in TM GA+DOX (vs. TM+GA and TM+DOX, $P>0.05$). Negative drug effects were observed in cortical bone (cross-sectional volume, cortical volume, marrow volume; $P<0.05$), but combined GA+DOX did not exacerbate these effects. Additionally, there were no protective cortical bone effects observed in TM. **Conclusions:** Combined GA+DOX exacerbates cancellous osteopenia in the tibia, and treadmill running provided only minor protection.

Keywords: Anthracycline, Chemotherapy, Estrogens, Exercise, Osteopenia

Introduction

The anthracycline antibiotic doxorubicin (DOX, trade name Adriamycin[®]) is used as an effective treatment for a variety of cancers. Although DOX is associated with a myriad of negative side effects and toxicities, one clinical concern is ovarian damage which may progress to irreversible sterility^{1,2}. This ovarian damage is characterized by DOX accumulating first in the centrally located stromal cells followed by a gradual accumulation toward the periphery resulting in apoptosis³. One approach to minimizing DOX-induced ovarian damage is to treat patients with a gonadotropin releasing hormone (GnRH)

analogue such as goserelin acetate (GA) prior to DOX treatment. GA administration suppresses ovarian function which in turn reduces the susceptibility to DOX-induced damage⁴⁻⁶. Upon termination of DOX treatment, GA administration is also terminated, and ovarian function returns to normal^{1,7}.

An additional concern facing patients receiving either GA or DOX treatment is bone loss. Estrogen levels fall to postmenopausal status with GA treatment, and the negative effects of low estrogen availability on bone have been well established (for Review see Ref 8). We have reported previously that 8 weeks of GA treatment in the female rat has a dramatic impact on cancellous bone measured at the proximal tibia⁹. Similarly, DOX is known to have osteotoxic effects with treatment reducing bone mineral content and bone mineral density¹⁰. Although these treatments administered alone have negative effects on bone, there is potential for combined GA and DOX treatment to induce a greater degree of bone loss than these treatments administered separately. With GA pretreatment before DOX (to preserve ovary function), the removal of osteoprotective estrogen may make the bone more susceptible to DOX-induced bone loss. However, there have been no studies investigating the potential additive detrimental effects of GA and DOX on

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	Week of Training							
	1	2	3	4	5	6	7	8
Duration (min)	20	40	60	60	60	60	60	60
Speed (m/min)	25	25	25	25	18	18	18	18
% Grade	0	0	0	3	6	9	12	15

Table 1. Treadmill training protocol.

bone. Therefore, the first purpose of this study was to examine the effects of combined GA and DOX treatment on bone when compared to GA and DOX treatments alone. It was hypothesized that GA treatment prior to DOX administration would exacerbate the detrimental skeletal effects of DOX.

Approaches to minimize cancer treatment-related bone loss have been investigated and include the use of bisphosphonates, vitamin D, and calcium supplementation¹¹. Additionally, exercise has been proposed as a means of protecting the skeleton from cancer treatment-induced bone loss^{12,13}. It has been shown, however, that voluntary wheel running provided very little protection against GA-induced tibial cancellous osteopenia in female rats⁹, and the lack of osteoprotection was speculated to be due to the sporadic and low-intensity nature of voluntary wheel running. Additionally, it has been shown that voluntary wheel running did not protect against DOX-induced bone degeneration in the juvenile rat¹⁴. In examining the results of these two studies, it is plausible that a more intense and regimented, load bearing type of exercise than voluntary wheel running might be necessary to attenuate GA- and DOX-induced bone loss. Therefore, the second purpose of this study was to examine if motorized treadmill running would provide protection against the detrimental effects of GA-, DOX-, and combined GA and DOX-induced osteopenia. It was hypothesized that treadmill training would provide osteoprotection against the detrimental effects of GA-, DOX-, and combination GA and DOX therapy on bone.

Materials and methods

Animals, drug administration, and exercise. The University of Northern Colorado's Institutional Animal Care and Use Committee approved all procedures which were in compliance with the Animal Welfare Act guidelines. All bones analyzed were obtained from animals included in a previously published study examining the effects of treadmill training on combined GA and DOX-induced cardiac dysfunction¹⁵, and all bones were excised post mortem following cardiac function analyses. In brief, 10-week old female Sprague-Dawley rats (n=80) purchased from Harlan (Indianapolis, IN) were maintained on a 12:12 hr light:dark cycle in an environmentally controlled facility and allowed access to standard chow and water *ad libitum*. Animals were randomly assigned to one of the following groups: 1) combined GA and DOX (GA+DOX, n=20), 2) GA and saline (GA+SAL, n=20), 3) control and DOX (CON+DOX, n=20), or

4) control and saline (CON+SAL, n=20).

GA+DOX received one implant of Zoladex[®] (3.6 mg GA housed in a 5 mm long x 1 mm wide biodegradable cylinder, AstraZeneca, Macclesfield, Cheshire, UK) on day 1, implanted s.c. at the scruff of the neck. Each 3.6 mg GA formulation of Zoladex[®] is effective at reducing estrogen synthesis for 28 days¹⁶. Beginning on day 15, animals received daily 1.5 mg/kg i.p. injections of 2 mg/mL DOX HCl for 10 consecutive days (cumulative 15 mg/kg). On day 29, animals received a second Zoladex[®] 3.6 mg GA implant.

GA+SAL animals received a s.c. Zoladex[®] implant at the scruff of the neck on day 1, and on day 15, they received daily i.p. 0.9% NaCl injections for 10 consecutive days at an equivalent volume of DOX HCl to act as sham injections. Then, on day 29, GA+SAL animals received a second s.c. Zoladex[®] implant at the scruff of the neck. Animals assigned to the CON+DOX group received a s.c. implant of biodegradable Silastic tubing (5 mm long x 1 mm wide, Dow Corning, Midland, MI) to act as a sham implant on day 1, and on day 15, they began receiving daily 1.5 mg/kg i.p. injections of 2 mg/mL DOX HCl for 10 consecutive days (cumulative 15 mg/kg). On day 29, CON+DOX animals then received a second Silastic tubing sham implant. CON+SAL animals received a s.c. Silastic tubing sham implant on day 1 and day 29, and on day 15, they received i.p. 0.9% NaCl injections for 10 consecutive days at an equivalent volume of DOX HCl to act as a sham treatment.

Animals were also randomized to treadmill training (TM) or sedentary (SED) groups. TM animals (TM GA+DOX, n=10; TM GA+SAL, n=10; TM CON+DOX, n=10; TM CON+SAL, n=10) began a progressive motorized treadmill protocol (Table 1) 24 hours following the initial GA or CON implant (day 1). TM animals trained 5 d/week throughout the entire treatment period (8 weeks). SED animals (SED GA+DOX, n=10; SED GA+SAL, n=10; SED CON+DOX, n=10; SED CON+SAL, n=10) were limited to normal cage activity throughout the 8 week treatment period.

Bone architecture analysis. On day 57, tibiae were excised and placed in 70% ethanol. Micro computed tomography (μ CT) was used for nondestructive three-dimensional evaluation of cancellous and cortical bone architecture. The tibiae were scanned in 70% ethanol at a voxel size of 16 x 16 x 16 μ m using a Scanco μ CT40 scanner (Scanco Medical AG, Basserdorf, Switzerland). The threshold was determined empirically and set at 245 (0-1,000 range) for cancellous bone in the proximal tibial metaphysis and for cortical bone in the tibial diaphysis. Seventy five slices (1200

	CON+SAL		GA+SAL		CON+DOX		GA+DOX	
	SED	TM	SED	TM	SED	TM	SED	TM
1 st Implant BM (Day 1, g)	209±9	212±5	216±4	212±4	218±5	218±8	209±13	216±5
Initial Injection BM (Day 15, g)*	229±10	236±3	262±5†	251±4	239±5‡	241±5	259±9†	251±5
2 nd Implant BM (Day 29, g)*	236±8	251±4	293±4†	276±3†	235±5‡	240±7‡	253±9†‡	247±7‡
Final BM (Day 57, g)*	263±10	266±8	327±7†	310±5†	295±16‡	295±8	327±9†	290±11

Data are means ± SEM. CON, control implant; SAL, saline; GA, goserelin acetate; DOX, doxorubicin; SED, sedentary; TM, treadmill; BM, body mass.
* Significant drug effect ($P<0.05$)
† $P<0.05$ vs. activity-matched CON+SAL
‡ $P<0.05$ vs. activity-matched GA+SAL

Table 2. Body masses.

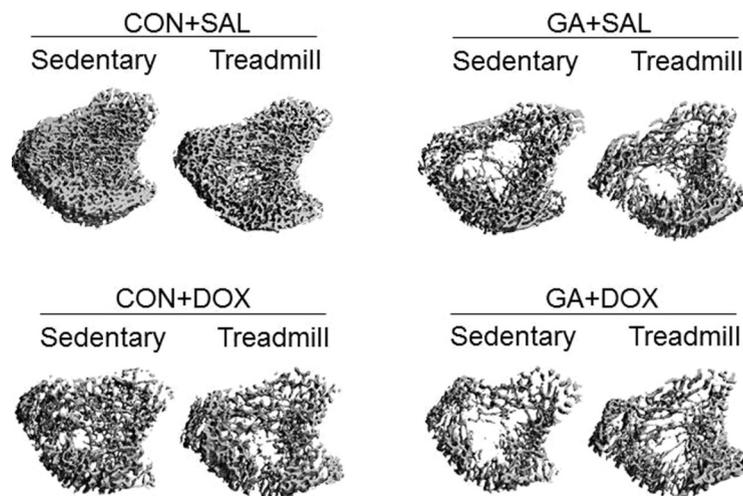


Figure 1. Representative images of cancellous bone in proximal tibia metaphysis. CON, control implants; SAL, saline injections; GA, goserelin acetate implants, DOX, doxorubicin injections.

(μm) were evaluated in the proximal tibia with the volume of interest being 1 mm below the growth plate which included only secondary spongiosa. Direct cancellous bone measurements included: 1) cancellous bone volume/total tissue volume (the volume of interest occupied by cancellous bone, %), 2) trabecular number (number of trabeculae, 1/mm), 3) trabecular thickness (mean thickness of individual trabeculae, μm), and 4) and trabecular spacing (the distance between trabeculae, μm). For assessment of cortical bone, 20 slices (320 μm) were evaluated in the tibial midshaft. Direct cortical bone measurements included: 1) cross-sectional volume (combined volume of cortical bone and bone marrow, mm^3), 2) cortical volume (volume of cortical bone, mm^3), 3) marrow volume (volume of bone marrow, mm^3), and 4) cortical thickness (the width of the cortical bone, μm).

Statistical analysis. Data are presented as mean ± SEM. A two-way (activity x drug) analysis of variance was performed on each variable to determine main effects and interactions.

When a significant main effect or interaction was observed, Bonferroni *post hoc* testing was performed to identify where differences existed. Significance level was set at $\alpha=0.05$.

Results

Body masses obtained throughout the 57 day experimental period are presented in Table 2. At the time of the initial GA or CON implant (day 1), there were no body mass differences among groups. However, at the time of the first DOX or SAL injection (day 15), a significant drug main effect was observed ($P<0.0001$), and *post hoc* analysis revealed that sedentary animals receiving GA implants had significantly higher body masses than animals receiving CON implants ($P<0.05$). At the time of the second GA or CON implant (day 29), a significant drug main effect was observed ($P<0.0001$) with SED GA+SAL having a higher body mass than SED CON+SAL

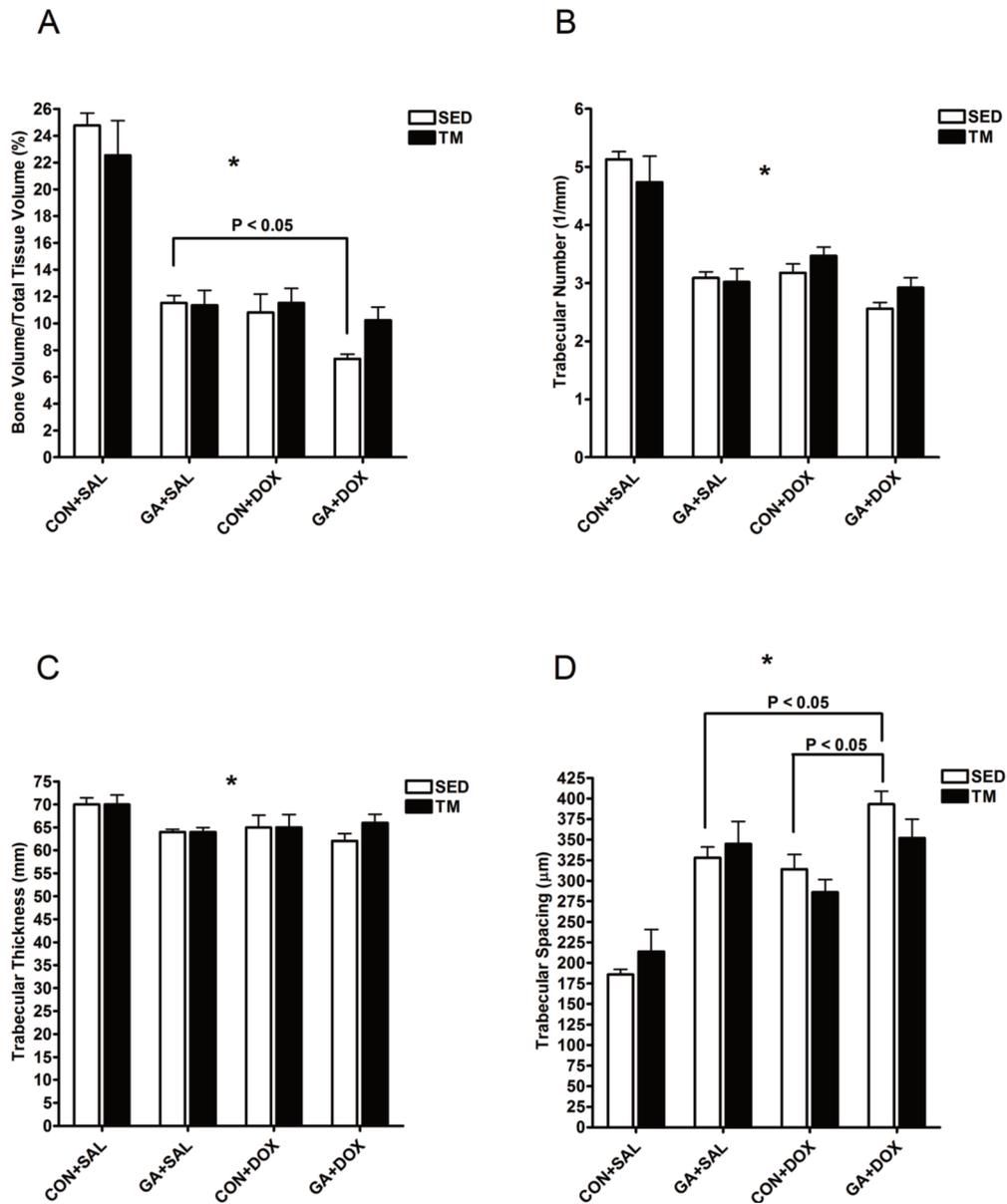


Figure 2. Tibia cancellous bone parameters. A, bone volume/tissue volume; B, trabecular number; C, trabecular thickness; D, trabecular spacing. CON, control implants; SAL, saline injections; GA, goserelin acetate implants, DOX, doxorubicin injections. * significant drug effect ($P < 0.05$).

SED CON+DOX, and SED GA+DOX ($P < 0.05$) and TM GA+SAL having a higher body mass than TM CON+SAL, TM CON+DOX, and TM GA+DOX ($P < 0.05$). In addition, SED GA+DOX had a significantly higher body mass than SED CON+SAL ($P < 0.05$). Likewise, a significant drug effect ($P < 0.05$) was observed for body mass at the time of sacrifice (day 57) with GA+SAL having a significantly higher body mass than activity-matched CON+SAL ($P < 0.05$) and SED GA+DOX having a higher body mass than SED+CON SAL ($P < 0.05$). Additionally, SED GA+SAL final body mass was higher than SED CON+DOX final body mass ($P < 0.05$), and SED GA+DOX had a higher final body mass than SED CON+SAL ($P < 0.05$).

Representative images of cancellous bone in the proximal tibia for each experimental group are illustrated in Figure 1, and measured cancellous bone parameters are presented in Figure 2. For each cancellous bone endpoint (bone volume/tissue volume, trabecular number, trabecular thickness, and trabecular spacing), significant drug effects ($P < 0.05$) were observed but there were no activity effects or drug x activity interactions for these measures (Figure 2). With *post hoc* testing, SED GA+DOX had a significantly lower bone volume/tissue volume than SED GA+SAL ($P < 0.05$, Figure 2A), and SED GA+DOX had significantly greater trabecular spacing than SED GA+SAL and SED CON+DOX ($P < 0.05$, Figure 2D). This suggests an additive effect of combined GA+DOX on

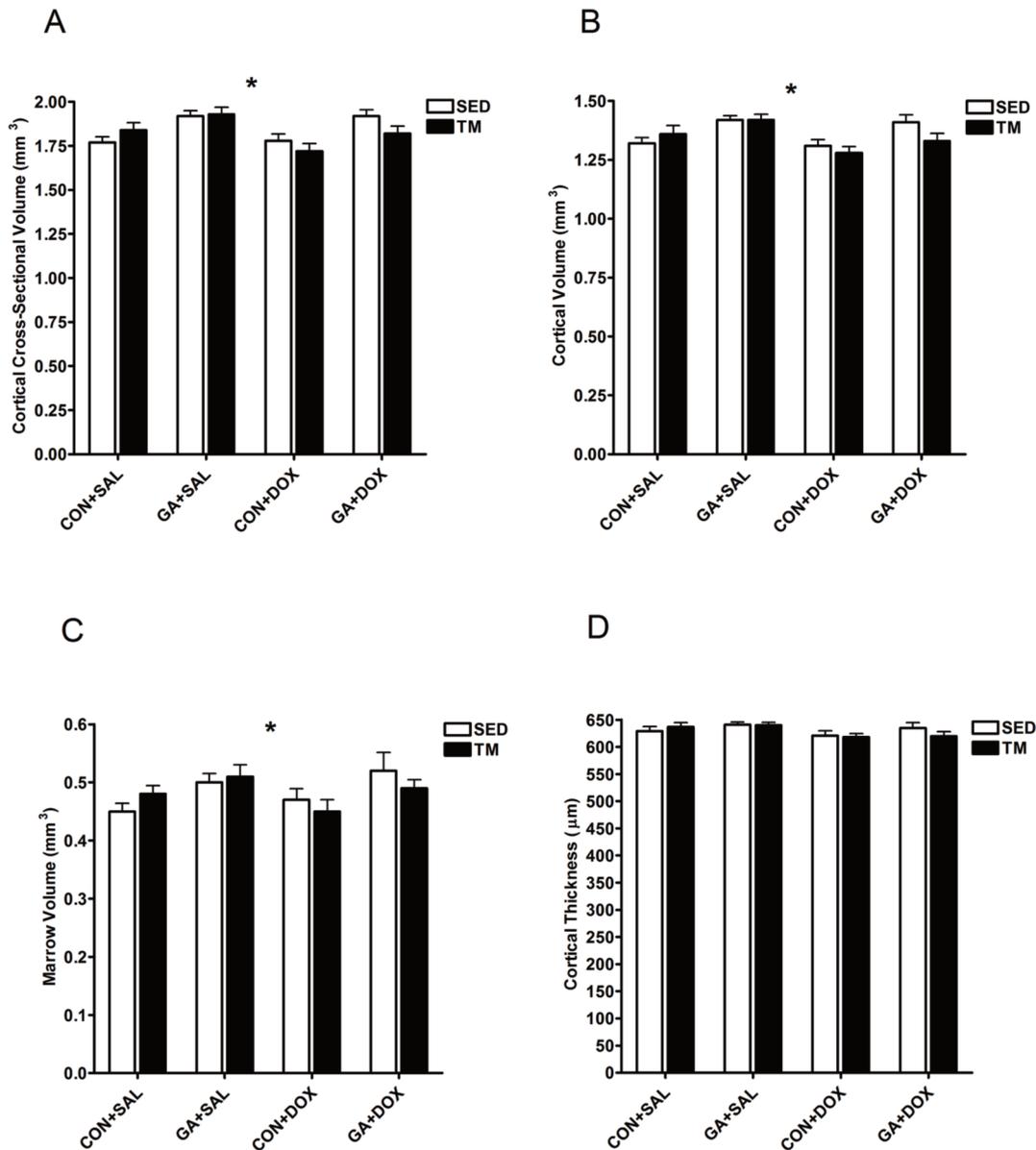


Figure 3. Cortical bone parameters. A, cross-sectional volume; B, cortical volume ; C, marrow volume; D, cortical thickness. CON, control implants; SAL, saline injections; GA, goserelin acetate implants, DOX, doxorubicin injections. * significant drug effect ($P < 0.05$).

bone volume/tissue volume when compared to GA treatment alone and trabecular spacing when compared to GA and DOX treatments alone in sedentary animals. These significant GA+DOX-induced bone volume/tissue volume and trabecular spacing differences, however, were not observed in treadmill trained animals (TM GA+DOX vs. TM GA+SAL and TM CON+DOX).

Figure 3 illustrates cortical bone measures from each experimental group. Significant drug effects were observed for cortical cross sectional volume, cortical volume, and marrow volume ($P < 0.05$, Figures 3A, 3B, and 3C, respectively). No activity effects or drug x activity interactions were observed for these parameters, and no individual group differences were

revealed with *post hoc* testing. No drug/activity main effects or interactions were observed for cortical thickness ($P > 0.05$, Figure 3D).

Discussion

The current study examined the effects of combined GA and DOX treatment on bone architecture of the tibia and whether treadmill running during treatment provided osteoprotection. All three drug treatments (GA, DOX, and GA+DOX) lead to osteopenia-like alterations in trabecular bone in the metaphysis (i.e., significant drug main effects), but not cortical bone in the

diaphysis. Overall, the cancellous parameters bone volume/tissue volume and trabecular number were lower and trabecular spacing was higher by ~50% with treatments when compared to sedentary controls (Figure 2A, B, and D), and the same degree of treatment-induced bone alteration was not observed in cortical bone (Figure 3A, B, and C).

Although the cancer treatments, alone and in combination, had an impact on the skeleton, the effects that treatments had on body mass are also worthy of note. GA treatment has been previously shown to increase body mass in the female rat using a similar dosing scheme¹⁷, and GA treatment is associated with increased fat mass and decreased lean mass¹⁸. DOX treatment typically results in a decrease in body mass especially when administered as a large bolus doses¹⁹. However, in the current study, the dose was spread over the course of ten consecutive days, and as such body mass for CON+DOX animals did not increase in the first 14 days (i.e., day 15 to 29) whereas CON+SAL body masses increased by a mean of 11 grams. In the following 28 days, body masses did increase in CON+DOX, and some of this weight gain could potentially be due to ascites as peritoneal fluid has been shown to increase with DOX treatment²⁰. Combining DOX with GA tended to blunt the overall weight gain associated with GA treatment, and this attenuation was more pronounced in the TM GA+DOX group. Although body composition was not analyzed in the current study, it is likely that some of the weight gain was due to increased fat mass since one of the elements of fat free mass (i.e., bone) was degraded. However, treadmill running ameliorated the weight gain in GA+CON and GA+DOX groups suggesting protection against increases in fat.

The additive effects of GA+DOX when compared to GA and/or DOX alone were only observed in two of the measured cancellous bone parameters. SED GA+DOX had a significantly lower bone volume/tissue volume than SED GA+SAL and significantly higher trabecular spacing than SED GA+SAL and SED CON+DOX. The protective effects of exercise on GA+DOX treatment were minor. No activity main effects or interactions were observed, but the aforementioned significant cancellous bone changes observed in SED GA+DOX when compared to SED GA+SAL and SED CON+DOX were not observed between TM GA+DOX, TM GA+SAL, and TM CON+DOX. Although not sufficient to prevent osteopenia, the similarities between treadmill-trained GA, DOX, and GA+DOX groups suggest a modest level of exercise-induced cancellous bone preservation in the tibia.

The negative effects of GA treatment alone on bone stem from the withdrawal of estrogen from the system. Estrogens are instrumental in bone maintenance as they regulate bone turnover balance²¹, and estrogen removal results in a net increase in bone resorption^{22,23}. The disruption in bone formation and resorption balance associated with estrogen withdrawal has become a clinical concern for many breast cancer patients receiving GnRH agonists²⁴, and approaches to minimize this bone loss have received considerable attention²⁵⁻²⁷ with load-bearing exercise being one such approach^{12,28}. However, the treadmill protocol used in the current study was not osteopro-

protective even though this protocol was shown previously to protect against GA+DOX-induced cardiac dysfunction²⁹. Since this exercise protocol provided protection against one treatment-related side effect (i.e., cardiac dysfunction), but not another (i.e., cancellous bone loss), the complexity of managing GA and DOX side effects becomes even more apparent.

It has been shown previously that substantial osteoprotective effects of load-bearing exercise may not be sufficient with estrogen depletion³⁰. Therefore, the use of alternative osteoprotective treatments such as bisphosphonates³¹ and vitamin D³² may be necessary to help counteract GA-induced bone loss. It has been reported, however, that no additive osteoprotective effects of treadmill training combined with zoledronic acid treatment (a bisphosphonate) were observed in ovariectomized rats³³ suggesting that addressing GA-induced bone loss may be more complex than combining two or more potentially osteoprotective treatments (i.e., treadmill running and bisphosphonates). The exercise mode itself may need to be examined as well, and it may be necessary for future studies to explore the effects of high-impact resistance training models (alone or in combination with other treatments) on GA-induced bone loss.

DOX may interfere with the normal cell cycle in bone cells. By intercalating DNA, inhibiting topoisomerase II and helicase activities, and generating reactive oxygen species, DOX is effective at halting the growth and proliferation of a variety of malignant cells, but these mechanisms may also be responsible for the disruption of normal bone metabolism³⁴. A well-known mechanism of DOX toxicity is its interaction with mitochondria which results in increased reactive oxygen species formation thereby causing cellular damage. It is possible that DOX's interaction with osteocyte mitochondria results in oxygen radical-induced damage. Furthermore, since DOX also induces ovarian damage², the impact that reduced estrogen production has on bone following DOX treatment cannot be overlooked.

We showed previously that voluntary wheel running did not protect against osteopenia in the juvenile rat receiving DOX¹⁴, and treadmill running in the current study did not protect against DOX-induced osteopenia in a more mature skeleton than that of the juvenile rat study. Exercise has been shown to be an effective approach to ameliorating chemotherapy-induced bone loss^{12,35}, but these clinical studies typically include patients receiving a combination of anticancer drugs which may or may not include DOX. Nonetheless, cancer patients receiving chemotherapy have a higher rate of osteopenia and osteoporosis than healthy controls³⁴, but it is hard to determine whether this bone loss is due to the anticancer drugs, a more sedentary lifestyle following treatment (due to increased fatigue), or a combination of both. The model employed in the current study addressed these effects individually and in combination, and exercise was not protective against DOX-induced osteopenia. Once again, it is necessary for future studies to examine the effects of combination treatments such as bisphosphonates, vitamin D supplementation, and exercise with higher mechanical loading (i.e., resistance training) on DOX-induced bone loss.

Combining GA and DOX in a clinically-relevant manner (i.e., downregulating ovarian function with GA treatment prior to and during DOX administration) resulted in significant cancellous osteopenia (decreased bone volume/tissue volume and increased trabecular spacing) when compared to GA and DOX treatments alone. To our knowledge, the current study is the first to demonstrate exacerbated detrimental effects on bone with combined GA and DOX treatment. The additive effects of the two drugs on bone may be due to their effects on osteoblasts and osteoclasts. Not only do estrogens regulate osteoblast and osteoclast production and apoptosis²¹⁻²³, but DOX administration results in a dose-dependent reduction in osteoblast viability³⁶ and an increase in osteoclast-lined bone perimeter³⁷. These combined osteoblast and osteoclast effects of estrogen withdrawal and DOX treatment interfere with the normal bone turnover process which would be a clinical concern for cancer patients receiving such a treatment regimen. Treadmill exercise promoted a mild protective effect against this combined treatment-induced osteopenia as treadmill trained GA+DOX animals possessed bone volume/tissue volume percentages similar to those of animals receiving GA or DOX.

It would be necessary for future studies to investigate if the protective effects of exercise stem from osteoblast activation, osteoclast suppression, or a combination of both. Furthermore, effects of exercise on mechanical properties and bone formation (i.e., calcein labeling) in GA+DOX treated rats should be analyzed to examine if these properties are altered with treatments. Future work should also examine if resistance training, vitamin D administration, and bisphosphonates (administered alone or in combination) can provide a greater level of osteoprotection. It is important to mention that even though the current study is the first to investigate GA+DOX's combined effects on bone, it was limited to only analyzing tibiae. Femur and vertebra have been shown to be impacted by estrogen withdrawal³⁸ and DOX treatment³⁹, and these sites should be analyzed in future studies. Providing a more global assessment of cancer treatment-effects on the skeleton (as well as osteoprotective approaches) will help better understand and manage treatment-related side effects. For example, breast cancer patients are at increased risk of fractures due to treatment-related side effects on bone^{40,41}, and since the current study found that combined GA+DOX resulted in decreased bone volume/tissue volume and increased trabecular spacing in trabecular bone in the metaphysis when compared to GA or DOX administered alone, it is necessary to consider the combined treatment's effects on bone in a clinical setting. In addition, patients receiving treatments for prostate cancer that include androgen deprivation therapy (which may be accomplished using GA) are also at an increased risk of fractures⁴² which potentially could be exacerbated when combined with DOX, and thus, there is also a need to further investigate treatment and exercise effects on the male skeleton.

In conclusion, GA and DOX administered alone promoted bone degradation in the trabecular (cancellous) area of the tibia, and treadmill training during these individual treatments was not osteoprotective. Combined GA and DOX treatments

resulted in greater osteopenia in tibial cancellous bone when compared to GA and DOX treatments alone, and treadmill exercise during this combined treatment provided only minimal protection. This lack of overall protection suggests that treadmill training is not an effective intervention against combined GA and DOX-induced osteoporosis, and additional approaches are needed to provide protection.

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