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RESEARCH ARTICLE

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Acetaminophen influences musculoskeletal signaling but not adaptations to endurance exercise training

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Abstract

Acetaminophen (ACE) is a widely used analgesic and antipyretic drug with various applications, from pain relief to fever reduction. Recent studies have reported equivocal effects of habitual ACE intake on exercise performance, muscle growth, and risks to bone health. Thus, this study aimed to assess the impact of a 6-week, low-dose ACE regimen on muscle and bone adaptations in exercising and non-exercising rats. Nine-week-old Wistar rats ($n = 40$) were randomized to an exercise or control (no exercise) condition with ACE or without (placebo). For the exercise condition, rats ran 5 days per week for 6 weeks at a 5% incline for 2 min at 15 cm/s, 2 min at 20 cm/s, and 26 min at 25 cm/s. A human equivalent dose of ACE was administered (379 mg/kg body weight) in drinking water and adjusted each week based on body weight. Food, water intake, and body weight were measured daily. At the beginning of week 6, animals in the exercise group completed a maximal treadmill test. At the end of week 6, rats were euthanized, and muscle cross-sectional area (CSA), fiber type, and signaling pathways were measured. Additionally, three-point bending and microcomputer tomography were measured in the femur. Follow-up experiments in human primary muscle cells were used to explore supraphysiological effects of ACE. Data were analyzed using a two-way ANOVA for treatment (ACE or placebo) and condition (exercise or non-exercise) for all animal outcomes. Data for cell culture experiments were analyzed via ANOVA. If omnibus significance was found in either ANOVA, a post hoc analysis was completed, and a Tukey's adjustment was used. ACE did not alter body weight, water intake, food intake, or treadmill performance ($p > .05$). There was a treatment-by-condition effect for Young's Modulus where placebo

Abbreviations: 4EBP1, Eukaryotic translation initiation factor 4E-binding protein 1; ACE, acetaminophen; AKT, protein kinase B; AMPK, 5′ AMP-activated protein kinase; BMD, bone mineral density; BV, bone volume; COX, cyclooxygenase; CSA, cross-sectional area; ERK, extracellular signal-regulated kinase; MHC, myosin heavy chain; microCT, micro computed topography; MMI, moment of inertia; NFATc1, nuclear factor of activated T cells; PPARGC1A, Peroxisome proliferator-activated receptor γ coactivator 1α; PTSG1, prostaglandin-endoperoxide synthase 1; PTSG2, prostaglandin-endoperoxide synthase 2; S6, S6 ribosomal protein; TV, trabecular volume.

exercise was significantly lower than placebo control ($p < .05$). There was no treatment by condition effects for microCT measures, muscle CSA, fiber type, or mRNA expression. Phosphorylated-AMPK was significantly increased with exercise ($p < .05$) and this was attenuated with ACE treatment. Furthermore, phospho-4EBP1 was depressed in the exercise group compared to the control ($p < .05$) and increased in the ACE control and ACE exercise group compared to placebo exercise ($p < .05$). A low dose of ACE did not influence chronic musculoskeletal adaptations in exercising rodents but acutely attenuated AMPK phosphorylation and 4EBP1 dephosphorylation post-exercise.

KEYWORDS

bone, muscle, muscle protein synthesis, NSAIDs, paracetamol, Tylenol

1 | INTRODUCTION

Acetaminophen ((N-acetyl-p-aminophenol, paracetamol) (ACE)) is an over-the-counter analgesic and antipyretic drug with a long-standing history of clinical applications[1,2] that is used by a wide range of people.[3–6] Although ACE's mechanism was once unclear, it is much like nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and celecoxib, because it inhibits cyclooxygenase (COX) enzymes.[7] Indeed, ACE has a higher affinity for COX-2 than COX-1 (4.4-fold).[8,9] ACE also affects central sites of action that differ from NSAIDs.[10] While the primary uses of ACE are to alleviate pain and reduce fever, research indicates that the physiological effects may influence musculoskeletal adaptations.[11,12] Understanding ACE's impact on musculoskeletal health is important, particularly for people who exercise or are susceptible to musculoskeletal injuries.[13–16]

ACE has multiple effects on muscle and skeletal structure that extend beyond its analgesic properties. Wu et al. found that chronic treatment with a very low dose of ACE (30 mg/kg of body weight per day(bw)) in rodents led to reduced myocyte apoptosis and increased muscle fiber size in aged muscles, possibly through the upregulation of myosin and actin expression.[17] These effects were also linked to reduced oxidative stress, including the lowering of superoxide levels and decreased protein translation.[17] Trappe et al. demonstrated that ACE (4000 mg/day) when combined with resistance training, enhanced muscle hypertrophy and strength in older adults without affecting liver or kidney function.[16] ACE consumption was not associated with changes in COX-1 and COX-2 expression yet resistance training resulted in a drug-independent increase in COX-1.[16] Additionally, this group found ACE (4000 mg/day) blunted post-exercise muscle protein synthesis rates after resistance exercise in young healthy adults, but not older adults, suggesting a possible negative effect on recovery in healthy muscle.[18] Others have found that ACE consumption (1000 mg/6 h) before resistance exercise suppresses the early response of the AKT pathway but has a negligible effect on the extracellular matrix in young healthy men.[12,19] On the other hand, findings with chronic low doses (1000 mg/day only on exercise days) taken by men ≥50 y for 16 weeks show no effect on muscle size or bone biomarkers.[20]

During endurance exercise, pain tolerance is positively correlated with performance; thus, increasing the pain threshold with ACE ingestion could lead to improved performance.[21] For example, recreational runners completing a 3-kilometer time trial improved performance following ingestion of 1500 mg of ACE ingestion compared to a placebo.[22] Indeed, there seem to be positive effects of ACE on endurance performance in some,[23–25] but not all studies.[26,27] A recent meta-analysis found that ACE enhanced performance by a trivial to small magnitude in time-to-exhaustion endurance tests, but not in time trials.[13] However, despite ACE displaying a multitude of effects on muscle and skeletal structures, potentially impacting muscle recovery and endurance performance, the underlying molecular mechanisms driving changes in the musculoskeletal system remain largely uncharacterized.

Exercise is important for bone development and health.[28] Rodent models with various training modalities, such as treadmill running, have been used to investigate the effects and mechanisms of exercise on bone.[29–31] Recent investigations have also revealed interactions between ACE with bone healing and bone structural integrity. Indeed, NSAIDs may have a detrimental impact on bone health by slowing down the healing process, disrupting callus formation, and compromising the mechanical properties of bones, which in turn elevates

the likelihood of nonunion and may inhibit bone fracture healing.[32,33] Other studies indicate that ACE may increase the risk of stress fracture, raising concerns about chronic effects on bone health.[34,35] Taken together, These observations suggest that ACE may have adverse effects on various aspects of bone physiology and bone health.[36]

A limitation to studying drugs in animals and humans is dosage. The no observed adverse effect level (NOAEL), used in toxicology, is the highest dose where the effects observed by a drug do not adversely affect a subject. The current NOAEL for ACE is 500 mg/kg/bw in rats[37] and the upper limit of prescription in humans is 4000 mg/day although knowledge about appropriate ACE usage and doses is poor in the general population—leading to misuse.[38] Interestingly, research has indicated that low doses of ACE have important effects on human physiology.[39,40] Thus, it is important to study low doses, which could mimic chronic ACE ingestion. However, to our knowledge, only one study has studied the effects of ACE at low dosages (75 mg/kg/bw) in rodents, finding that ACE ameliorated muscular mechanical hyperalgesia when developed after a model lengthening contraction (LC) in hindlimb muscles.[41] A method to characterize a range of doses that extend beyond the NOAEL is cell culture, which we have previously used to characterize effects of arachidonic acid and NSAIDs in primary human cell culture.[42,43]

This study aimed to assess the impact of a 6-week, low-dose ACE treatment on muscle and bone adaptations in exercising and non-exercising rats using a treadmill. To better understand ACE dosages above the NOAEL, we also used a cell culture model to test muscle viability, growth, and myotube fusion. Our hypothesis was that ACE would attenuate the chronic adaptations in muscle and bone induced by exercise in rodents and that ACE concentrations above 65 μM in cell culture, which is a comparable concentration in humans taking ACE, would be detrimental to myoblast viability and myotube fusion.

2 | METHODS

All procedures were conducted under the guidelines of the institutional animal care and use committee (IACUC) at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA, USA). Male adult Wistar Rats ($n = 40$) were purchased weighing 175 g and received at about 8–10 weeks of age and were acclimated for ~2 weeks within the vivarium at USARIEM. Rats were housed individually in cages with running wheels and allowed ad libitum access to food and water.

2.1 | Exercise

Before the experimental training period, the rats were introduced to a 5-lane running treadmill (#76-0895, Panlab Harvard Lab Apparatus, Holliston, MA, USA) as part of a 1-week familiarization protocol: on days one and two, the rats sat on a stationary treadmill for 15 min; on day 3, the rats were run at 15 cm/s for 15 min at a 5% incline; on day 4, the rats were run for 2 min at 15 cm/s and 18 min at 20 cm/s at a 5% incline; and on day 5 the rats were run for 2 min at 15 cm/s, 26 min at 20 cm/s, and 2 min at 25 cm/s at a 5% incline. During the training period, the rats who exercised ran 5 days per week for 6 weeks for 2 min at 15 cm/s (9 m/min), 2 min at 20 cm/s (12 m/min), and 26 min at 25 cm/s (15 m/min), all at a 5% incline. To encourage running, a shock from an electric grid at the rear of the treadmill or intermittent air puffs to the hindquarters were administered as needed to encourage the rats to run on the treadmill. Controls were placed on a stationary treadmill for an equivalent amount of time during the familiarization week and experiment weeks. Upon completion of the 6-week training or non-training period, rodents were rapidly anesthetized and euthanized within 30–45 min of their final bout of exercise or control procedures. Quadriceps was harvested, snap froze in liquid nitrogen and stored at −80°C until analyzed. Femurs were harvested and stored in gauze-soaked PBS at −20°C until analyzed.

2.2 | Maximal effort exercise test

Animals in the exercise group completed a maximal treadmill test on the first day of the sixth week of the training period.[44,45] This time point was selected to avoid confounding effects of acute maximal exercise performance on our primary outcomes that may have occurred with testing at the end of the 6-week training period. Briefly, rats in the exercise groups were placed on the treadmill and began a warm-up by running at 15 cm/s for 2 min, which was then increased to 20 cm/s for 2 min, and then increased to 25 cm/s for an additional 2 min. Following the warm-up period, the speed of the treadmill was incrementally increased by 5 cm/s (3 m/min) every 2 min until the test ended; throughout the testing protocol, the rats ran at a constant 5% incline. The performance test was stopped immediately when a rat received a cumulative shock stimulus (i.e., electrical stimulus; stimulus between 0 and 2 mA) of ≥30 s, at which point the time,

distance, and speed reached at the point of stoppage was recorded. Non-exercise groups were not tested since they were not habituated to treadmill running.

2.3 | NSAID administration

ACE was administered daily beginning the day prior to beginning the exercise protocol. For humans, the maximum recommended ACE dosage per day is 4000 mg. The rodent ACE dosage (379 mg/kg body weight) was calculated based on the human equivalent dose for a 65-kilogram adult (human equivalent dose (mg/kg) = animal dose (in mg/kg) × [animal Km/human Km] with animal Km = 6 and human Km = 37 and human equivalent dose = 4000 mg/65 kg adult), which was under the no-observed-adverse-effect level (NOAEL) for rodents.[37,46] The dosage was adjusted each week to account for changes in body weight. ACE was diluted into 400 mL of the rats drinking water and controls were given an equivalent volume of untreated water, and their bottles were changed on a matched schedule, and the amount of water consumed per day was recorded.

2.4 | Microcomputed tomography

To determine bone mass and geometry, each bone was scanned individually using a Bruker Skyscan 1275 microCT system equipped with a 1 mm aluminum filter using 55 kV and 181 μA scan settings and 74 ms of exposure time. Transverse scan slices were obtained by placing the long axis of the bone parallel to the *z*-axis of the scanner, using an isometric voxel size of 13 μm. Images were reconstructed using nRecon (Bruker) and analyzed using CTan (Bruker).

2.5 | Three-point bending

After microCT scanning was completed, the structural and mechanical properties of the extracted femur were quantified. The femur was oriented on a standard fixture with femoral condyles facing down. Next, a monotonic displacement ramp of 0.1 mm/s was applied until failure, with force and displacement acquired digitally. The force-displacement curves were converted to stress–strain using microCT-based geometry and analyzed using a custom GNU Octave script.

2.6 | Gene expression

RNA isolation, cDNA synthesis and RT-PCR were analyzed.[47] Tissue was removed under −80°C and placed on dry ice; RNA was extracted from the Quadricep muscles; 30 to 35 mg of muscle was cut and placed into Trizol (#15596018, Sigma Aldrich, St. Louis, MO, USA) then homogenized using a Bead Ruptor Elite homogenizer (#19-040E, Omni International, Kennesaw, GA, USA). The homogenate was centrifuged at 12 000*g* for 10 min at 4°C, and the supernatant was collected. 200 microliters of chloroform (#C2432, Sigma Aldrich, St. Louis, MO, USA) was added, and the samples were shaken for 15 s and then allowed to sit for 15 min. The samples were then centrifuged at 4°C for 10 min at 12 000 RPM, and the clear supernatant layer was collected. 500 microliters of isopropyl alcohol (#19516, Sigma Aldrich, St. Louis, MO, USA) was added, and the samples were vortexed and incubated overnight at −20°C. Samples were spun at 4°C for 15 min at 12 000 RPM and subsequent pellets were dried and then washed in 1 milliliter of 75% ethanol (#C2H60, Sigma Aldrich, St. Louis, MO, USA). Samples were then centrifuged at 4°C for 5 min at 7600 RPM, and pellets were dried until translucent and then resuspended in RNASE-free water. Samples were then heated in a heat block for 15 min at 60°C. RNA concentrations were determined using a Nanodrop 8000 (#ND8000, Thermofisher Scientific, Waltham, MA, USA). 40 μL of cDNA was created using 2 μg of RNA and the high-capacity cDNA reverse transcription kit (#4368814, Thermofisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions; a T100 Thermocycler (#1861096, Biorad, Hercules, CA, USA) was used to create the cDNA. 20 ng of cDNA was used to perform real-time PCR (RT-PCR) using an Applied Biosystems QuantStudio5 Thermocycler (Thermofisher Scientific, Waltham, MA, USA) with the following parameters: 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Measurements of ptgs1 (#Rn00566881_m1), ptgs2 (#Rn01483828_m1), myh2 (#Rn01470656_m1), myh3 (#Rn01332449_m1), myh7 (#Rn01488777_g1), mef2c (#Rn_01494040_m1), and NFATc1 (#Rn04280453_m1), all purchased from Thermofisher Scientific (Waltham, MA, USA) gene expressions were taken when the threshold of detection exceeded background (CT value) and was calculated using the $-2\Delta\Delta CT$ method and normalized to the level of 18 s (#4333760F, Thermofisher Scientific, Waltham, MA, USA) gene expression.

2.7 | Protein extraction and immunoblotting

Muscle tissue was removed from −80°C and placed on dry ice; 15 to 20 mg of muscle was cut and placed into 1× Lysis buffer and Halt protease/phosphatase inhibitor cocktail (#78446) and homogenized using a Bead Ruptor Elite homogenizer (#19-040E, Omni International,

Kennesaw, GA, USA). The homogenate was centrifuged at 12 000 *g* for 10 min at 4°C and the subsequent supernatant was collected and quantified by Bradford Assay to be used in western immunoblotting. Protein samples were run in 12-well 4%–20% tris-glycine gels (#XP04202BOX) for ~1.5 h at 125 V. Gels were transferred for 21 to 24 h at 27 V; once removed from transfer the membranes were washed in 1× tris buffered saline with tween (TBST) once for 5 min and then subsequently blocked in 5% non-fat dry milk (#1706404XTU) for at least 1 h. Membranes were then washed in 1× tris buffered saline with tween 3 times for at least 5 min each wash. Membranes were then incubated in primary antibody dilutes in 5% BSA and TBST overnight at 4°C on a tube rotator in the following antibodies: p-AKT S473 (#9271) (1:2000), Total AKT (#9272) (1:2000), p-AMPKα T172 (#2535) (1:1000), Total AMPKα (#2603) (1:2000), p-S6 S235/236 (#4858) (1:2000), p-S6 S240/244 (#2215) (1:2000), Total S6 (#2217) (1:2000), p-ERK 42/44 (#9101) (1:2000), Total ERK (#9102) (1:2000), GAPDH (#2118) (1:100 000), p-4EBP1 S65 (#9451) (1:1000), Total 4EBP1 (#9644) (1:2000), COX1 (#9896) (1:2000), and COX2 (#12282) (1:1000) all purchased from Cell Signaling Technology (Danvers, Ma, USA), Myosin Heavy Chain Fast Antibody (#M1570 (1:2000) Sigma Aldrich, St. Louis, MO, USA), Myosin Heavy Chain Slow (#ab11083, (1:2000) Abcam, Waltham, MA, USA), and Myosin Heavy Chain (#MAB4470, (1:5000) Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Membranes were then washed in 1 × TBST 3 times for 5 min each. Then incubated in either anti-Rabbit IgG antibody (#7074S), and anti-mouse IgG antibody (#7076) secondary antibody diluted in 5% BSA for up to 2 h. Next, membranes were washed in 1× TBST 3 times for 5 min, followed by incubation. Afterward, membranes were incubated in Super Signal chemiluminescent substrate (#34578) for 3 min and imaged using a Chemidoc XRS+ (#12003153, Biorad Hercules, CA, USA). Densiometric quantification analysis was done using NIH Image J 1.60.[48]

2.8 | Muscle immunohistochemistry

Muscle histology was conducted as previously described.[49,50] Briefly, soleus muscle was embedded in OCT after dissection then submerged in isopentane surrounded by liquid nitrogen for 15–30 s and stored at −80 until staining. For sectioning, the soleus was sliced into ~6–8 microns. Sections were fixed with 4% paraformaldehyde for 20 min at room temperature (RT), then a blocking buffer consisting of 5% goat serum, 2% BSA, and 0.5% Triton was applied for 120 min. The section was rinsed two times with phospho-buffered saline (PBS). Then, blocked with 5% goat serum at RT. Sections were then incubated with MHC 1 (1:100 w/v in 1% goat serum) for 30 min at RT. Next, sections were rinsed twice with PBS, then blocked with 5% goat serum at RT. Afterward, sections were incubated in Alexa 647 GAM (1:200 w/v in 1%) for 30 min at RT. The section was rinsed two times with PBS, then blocked with 5% goat serum for 30 min at room temperature. Sections were incubated in laminin (1:50 w/v in 1% goat serum) for 30 min at RT. Alexa 405 (1:200 w/v in 1% goat serum) was placed on the section for 30 min. Sections were then rinsed three times with PBS. MHC II solution was applied (1:100 w/v in 1% goat serum) for 30 min at 37°C. Slides were rinsed three times with PBS. Finally, prolonged gold was placed over each section followed by a cover slip and sections were imaged at 20× with a confocal microscope (Zeiss, Oberkochen, Germany).

2.9 | Cell culture

Experiments were conducted as previously published.[42,51,52] Human Skeletal Myoblasts (Cat #2580) were obtained from Lonza Technologies (Portsmouth, NH, USA), then grown and expanded in Skeletal Muscle Cell Growth Media and Bullet Kit (Cat #3245) at 37°C and 5% CO_2. Human Skeletal Myotubes were differentiated using low glucose Dulbecco's Modified Eagle Medium supplemented with 2% Horse Serum at 37°C and 5% CO_2. The 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Di phenyltetrazolium Bromide (MTT) proliferation assay (#30-1010 K) was purchased from ATCC (Manassas, VA, USA). Human skeletal muscle myoblasts were allowed to grow for 48 h in 65, 125, 250, or 500 μM of ACE and then the MTT assay was performed according to the manufacturer's instructions. For myotubes, an immunofluorescence antibody for MyHc (#MF-20) was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Images were derived from five randomly captured fields for each treatment group. The myotube fusion index was determined by counting the nuclei in every myotube (defined as MyHC-positive cells containing ≥2 nuclei) per field and dividing by the total number of nuclei in the field, as previously described.[53] Results are presented as means ± standard deviation from three independent experiments.

2.10 | Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) for treatment (ACE or placebo) and by condition (exercise or non-exercise) for all outcomes, except

maximal effort treadmill performance was analyzed via t-test. Data for cell culture experiments were analyzed via ANOVA. If omnibus significance was found for either ANOVA, a post hoc analysis was completed, and a Tukey's adjustment was used. Significance was considered at $p < .05$. Results are visualized using box and violin plots, with median and quartiles represented. Statistical analysis was performed with Graph Pad Prism 8.1.2. and R studio.

3 | RESULTS

3.1 | Body weight, water consumption, food consumption, and PGE metabolite

During the study, animals increased body weight (Figure 1A), but food and water intake were unchanged (Figure 1B,C, respectively). There was no treatment-by-time effect for body weight ($p > .05$); however, there was a significant effect of time ($p < .001$). There was no significant treatment by time effect for food intake or water intake ($p > .05$). Based on water intake, the animals consumed an average of 47.3–49.2 mL/day across the study translating to ~45 mg/kg/day of ACE. In humans, this is comparable to ~400 mg of ACE per day, which is slightly higher than the recommended intake for pain every 4–6 h (325 mg) in humans. Finally, there was no treatment by condition effect for urine PGE metabolite although this was only measured at endpoint ($p > .05$, Figure 1D). Together, this data indicates that ACE consumption did not alter body weight, water intake, or food intake, which could confound potential findings in muscle and bone.

3.2 | Treadmill exercise test

There was no significant difference between total distance (meters, $p = .574$, Figure 2A) or top speed (centimeters/

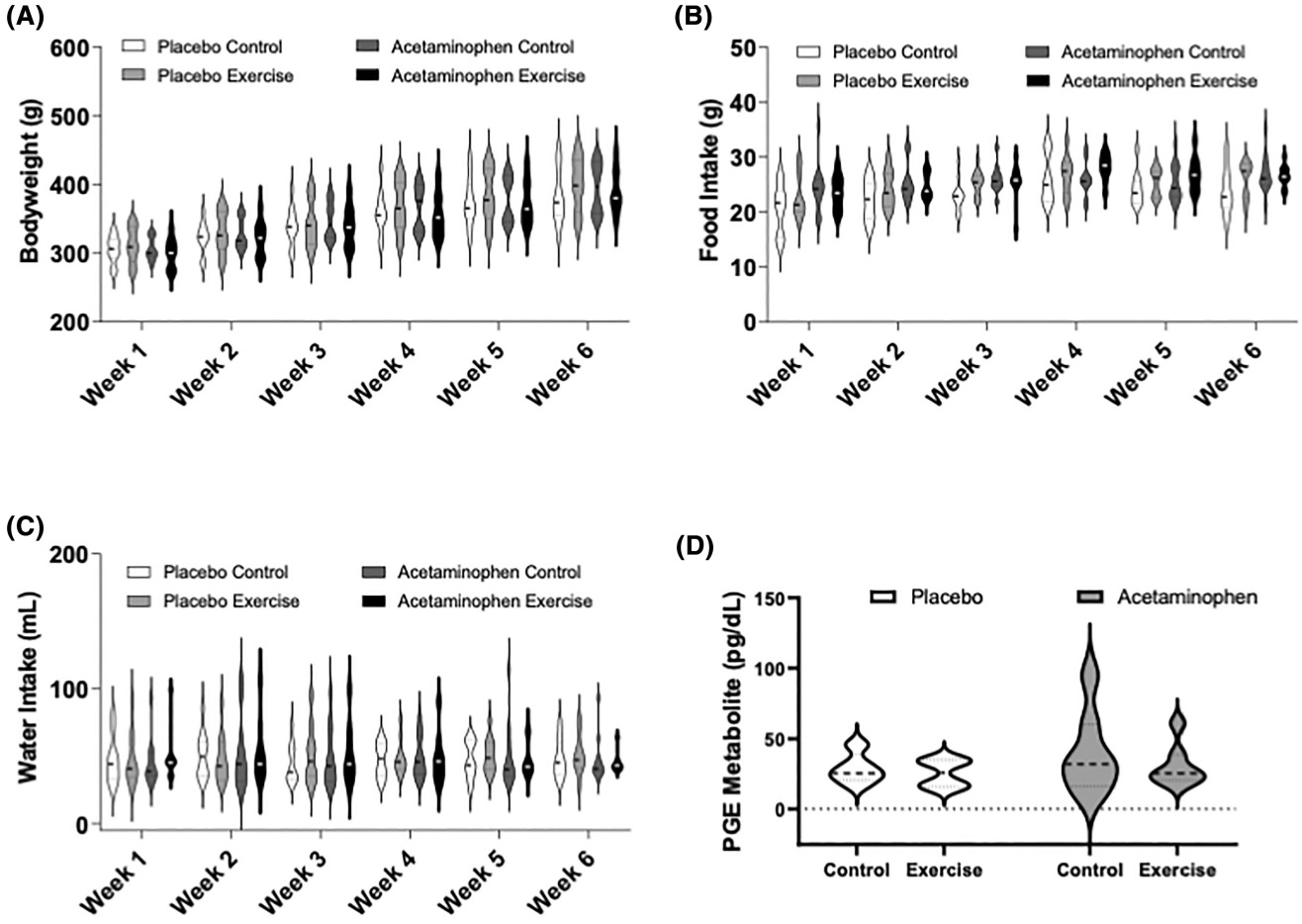

FIGURE 1 Bodyweight, food and water intake. Acetaminophen did not affect body weight, food and water intake, or urine prostaglandin E metabolite status. The male Wistar rat's daily (A) bodyweight, (B) food intake, and (C) water intake was tracked over the course of the six weeks. At the end of the six weeks, urine was collected and measured for (D) prostaglandin E metabolite. Data are representative of $n = 7$–8 per group and expressed as violin plots with medians.

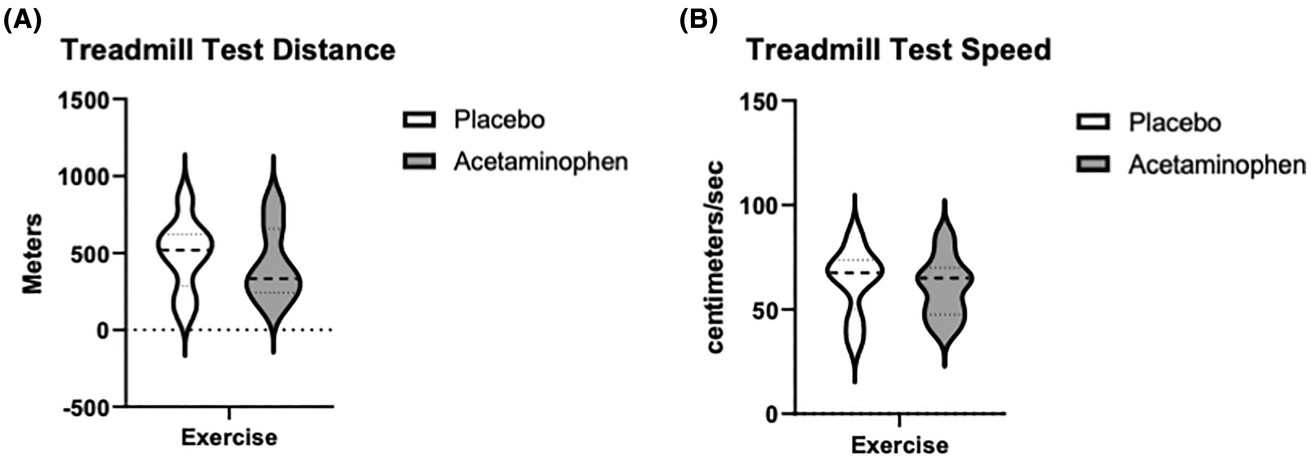

FIGURE 2 Treadmill performance test. Effects of chronic acetaminophen consumption on exercise performance. During the sixth week of the study, rats in the exercise groups completed a maximal treadmill test. Rats started by running at a speed of 15 cm/s (9 m/min) for 2 min, increased to 20 cm/s (12 m/min) for 2 min, and then increased by 5 cm/s every 2 min until the rat reached its maximum speed. At the end of the test the (A) total distance run over the course of the test and the (B) maximum speed were recorded. Data are representative of $n = 7–8$ per group and expressed as violin plots with medians.

second, $p = .642$, Figure 2B) between placebo and ACE exercise groups. This data indicates that ACE did not improve performance.

3.3 | MicroCT and 3-point bending

For 3 PB, there was no treatment by condition effect for ultimate moment ($p > .05$), bending rigidity ($p > .05$), ultimate stress ($p > .05$), ultimate displacement ($p > .05$), toughness ($p > .05$), post-yield toughness ($p > .05$), post-yield displacement ($p > .05$), or ultimate strain ($p > .05$) (Figure 3). There was a treatment-by-condition effect for Young's Modulus, and placebo control was significantly greater than placebo exercise (Figure 3D, $p = .04$). Young's Modulus defines elastic behavior. There was no treatment-by-condition effect for ultimate bending energy, but there was an exercise effect for ultimate bending energy (Figure 3F, $p = .04$). There was no treatment by condition effect for post-yield energy or post-yield strain (Figure 3G,K, $p > .05$), yet there was an exercise effect ($p = .049$ and $p = .045$, respectively).

For MicroCT, there was no treatment by condition effect for any outcome, including trabecular bone volume (bv)/ tissue volume (tv) ($p > .05$), trabecular bone mineral density (BMD) ($p > .05$), trabecular TV ($p > .05$), trabecular BV ($p > .05$), trabecular thickness ($p > .05$), trabecular number ($p > .05$), trabecular surface ($p > .05$), trabecular bone surface (BS)/TV ($p > .05$), trabecular bs/bv ($p > .05$), cortical TMD ($p > .05$), cortical T.Ar ($p > .05$), critical B.Ar ($p > .05$), cortical marrow area ($p > .05$), cortical mean eccentricity ($p > .05$), cortical Cs. Thickness ($p > .05$), cortical mean polar moment of inertia (MMI) ($p > .05$), or Cort B.Ar./T.Ar ($p > .05$) (Figures 4 and 5). This data indicates that ACE consumption did not alter bone adaptations with or without aerobic exercise training.

3.4 | Muscle size and fiber type

There was no treatment by condition effect for a pan-myosin heavy chain, myosin heavy chain fast, or myosin heavy chain slow (Figure 6A–C, $p > .05$). There was no treatment by condition effect for muscle fiber cross-sectional area (Figure 6D, $p > .05$). There was also no treatment by condition effect for muscle fiber type (Figure 6E, $p > .05$). This data indicates that neither ACE nor exercise changed the muscle phenotype.

3.5 | Muscle signaling

To determine if ACE influenced muscle signaling, we measured several canonical pathways that are changed with exercise. First, we measured myosin heavy chain with a pan-antibody in the quadriceps, finding no treatment by condition effect (Figure 7A, $p > .05$). Then we tested specific antibodies for myosin heavy chain slow and myosin heavy chain fast, also finding no treatment by condition effect (Figure 7B,C, $p > .05$). To determine if AMPK signaling was influenced, we measured AMPKα phosphorylation at the Threonine 172 site, finding treatment by condition

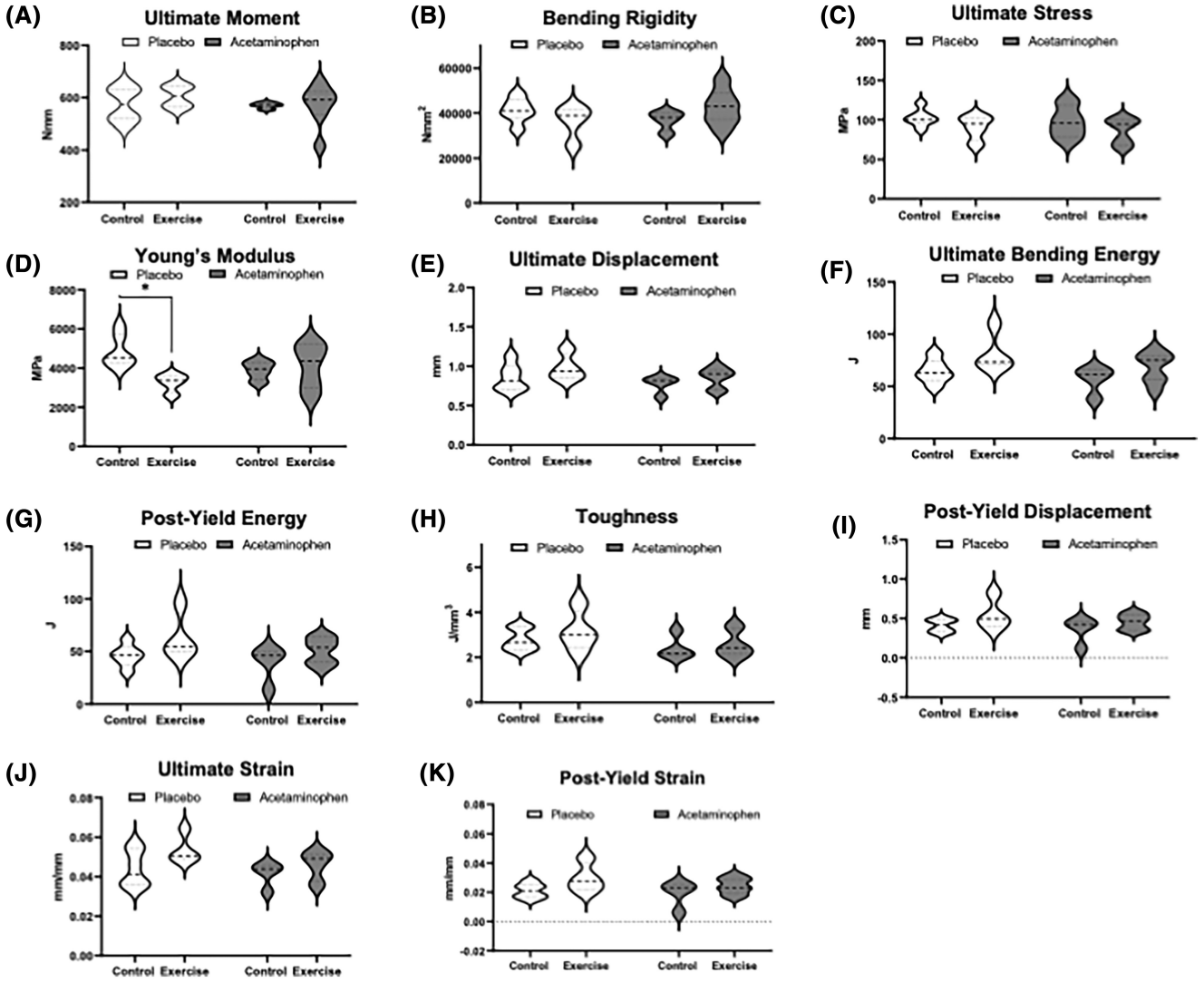

FIGURE 3 3-Point bending in the femur. Effects of acetaminophen on femur structural and mechanical properties. Structural and mechanical properties were measured via a three-point bending test (3PB). Femurs were placed with the femoral condyles facing down and a monotonic displacement ramp and 0.1 mm/s was applied until failure. The following measurements were obtained: (A) ultimate moment, (B) bending rigidity, (C) ultimate stress, (D) young's module, (E) ultimate displacement, (F) ultimate bending energy, (G) post-yield energy, (H) toughness, (I) post-yield displacement, (J) ultimate strain, and (K) post-yield strain. Data are representative of $n = 5–7$ per group and expressed as violin plots with medians. ($^*p < .05$).

effect (Figure 7D, $p = .036$). After post-hoc analysis, the placebo exercise group had higher p-AMPK T172 than the placebo control group ($p = .036$), but there was no exercise effect detected in the ACE-treated groups. Next, we measured p-ERK 42/44 (Figure 7E, $p = .996$), p-AKT S473 (Figure 7F, $p = .114$), p-S6 235/236 (Figure 7H, $p = .409$), p-S6 S240/244 (Figure 7I, $p = .531$), and COX-1 (Figure 7K, $p = .188$) finding no treatment by condition effects. However, when measuring p-4EBP1 S65, we found a significant treatment by condition effect (Figure 7G, $p = .0427$) with post-hoc analysis indicating a significantly lower phosphorylation in placebo exercise compared to placebo control ($p = .015$). Furthermore, ACE control was significantly higher than placebo exercise ($p = .006$) and ACE exercise ($p = .001$). Additionally, we found a treatment-by-condition effect for COX-2 (Figure 7L, $p = .0477$) and after post-hoc analysis, ACE control and ACE exercise group were different ($p = .0362$). We also found a treatment-by-condition effect for myogenin (Figure 7J, $p = .0164$) and after post-hoc analysis ACE control and ACE exercise groups were different ($p = .0136$). For mRNA expression, there was no treatment by condition effects for MYH2, MYH3, MYH7, PPARGC1a, Mef2c, NFATc1, or PTSG1 and PTSG2 (Figure 8A–H, all $p > .05$).

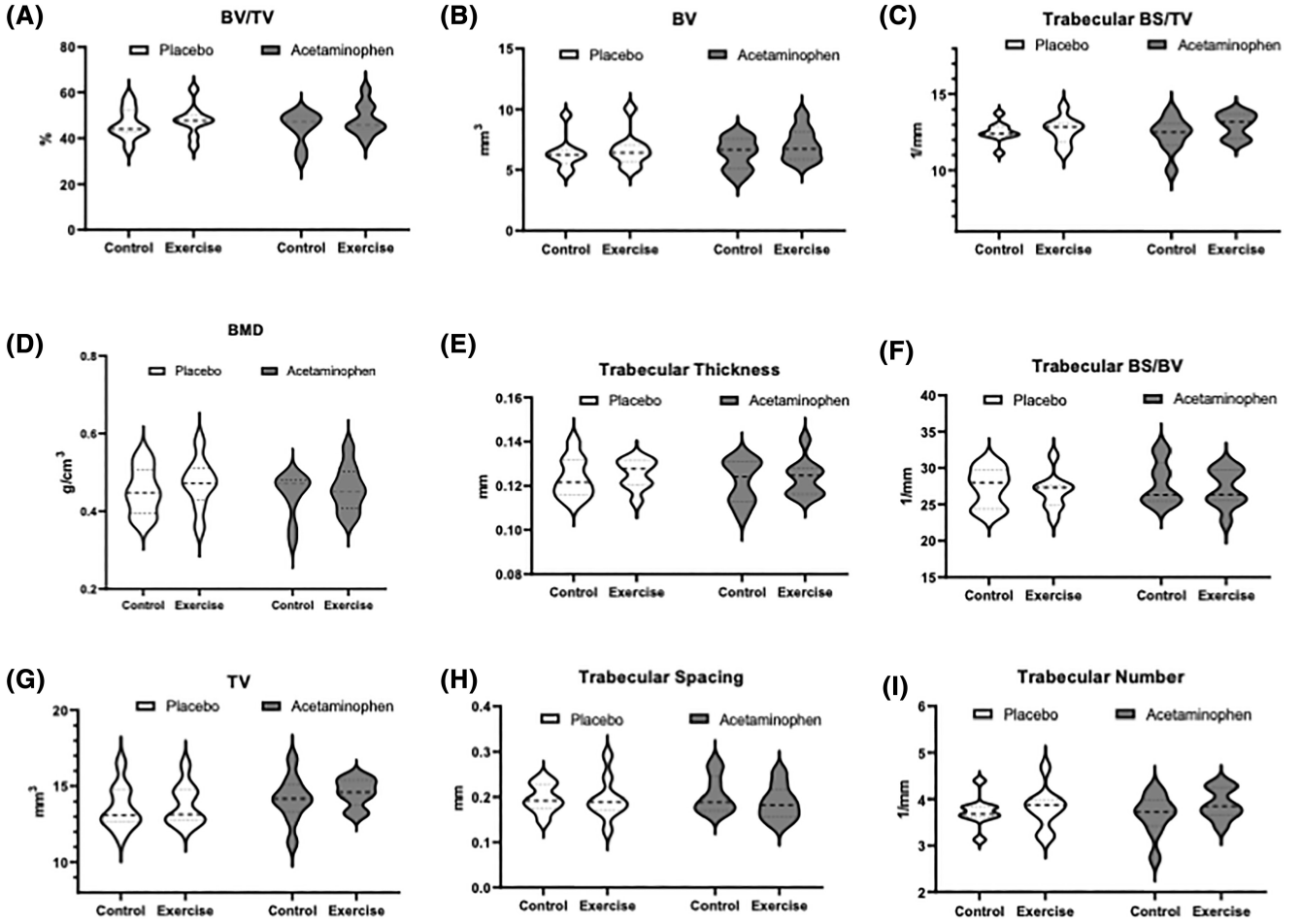

FIGURE 4 Trabecular geometry via MicroCT in the femur. Effects of acetaminophen on femur trabecular bone mass and geometry. Transverse femur slices were obtained using a Bruker Skyscan 1275 microCT utilizing a 1 mm aluminum filter using 55 kV and 181 μA scan setting with 74 ms of exposure time to obtain measurements for (A) bone volume/trabecular volume (BV/TV), (B) bone volume, (C) trabecular bone surface/tissue volume (BS/TV), (D) bone mineral density (BMD), (E) trabecular thickness, (F) trabecular bone surface/tissue volume (BS/TV), (G) tissue volume (TV), (H) trabecular spacing, and (I) trabecular number. Data are representative $n = 9$ per group and expressed as violin plots with medians.

3.6 | Cell culture

Next, we tested the effects of ACE in human muscle cell culture using a larger range to determine if higher concentrations would affect muscle cells. We found no significant difference in cell proliferation or myotube area when testing ACE concentrations from 65 to 500 μM (Figure 9, $p > .05$). The concentration of 65 μm represents the blood concentrations after consumption of ~400 mg of ACE, which is similar to our dose in the rodent experiments, although it is unknown if circulating levels translate directly to intramuscular levels.[54,55] Interestingly, in myotubes, we found that 200 μm of ACE, which is more than double the concentration seen in humans, reduced myotube fusion (Figure 9, $p < .05$) while lower concentrations did not have any effect compared to control cells. This data suggests that extremely high concentrations would have to be used to elicit negative adaptations in the skeletal muscles of humans or rodents.[56]

4 | DISCUSSION

The purpose of this study was to examine the influence of 6 weeks of daily ACE ingestion on skeletal muscle and bone adaptations following endurance exercise training in male Wistar rats. Our findings demonstrate that a low dose of ACE given to rodents while they were exercising on a treadmill for 6 weeks did not influence bone geometry. For bone strength, we found that Young's modulus, which is a measure of bone elasticity, was decreased with exercise and this change was attenuated by ACE while ultimate moment, bending rigidity, ultimate stress, and ultimate placement were not changed

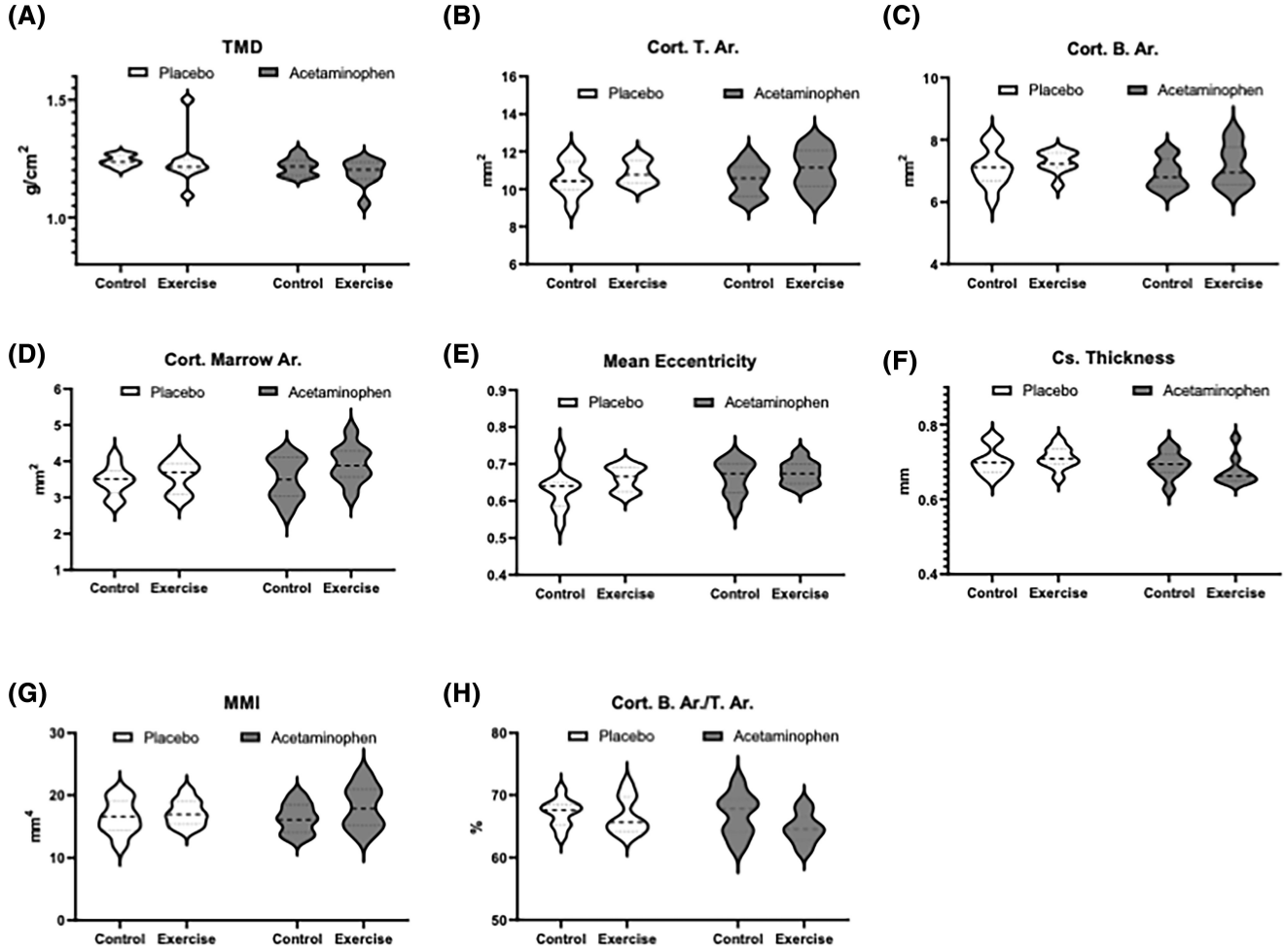

FIGURE 5 Cortical Geometry via MicroCT in the Femur. Effects of acetaminophen on femur cortical bone mass and geometry. Transverse femur slices were obtained using a Bruker Skyscan 1275 microCT utilizing a 1 mm aluminum filter using 55 kV and 181 μA scan setting with 74 ms of exposure time to obtain measurements for (A) tissue mineral density (TMD), (B) cortical tissue area (Cort. T. Ar.) (C) cortical bone area (Cort. B. Ar.), (D) cortical marrow area (Cort. Marrow Ar.), (E) Mean eccentricity, (F) cortical thickness, (cs. thickness) (G) mean polar moment of inertia (MMI), and (H) cortical bone area/trabecular area (cort. B. Ar./T. Ar.). Data are representative of $n = 9$ per group and expressed as violin plots with medians.

by either exercise or ACE. Furthermore, we found that ACE did not influence muscle fiber cross-sectional area or muscle fiber type. Upon inspection of molecular signaling in skeletal muscle, we found that the AMPK pathway was activated with exercise, but this effect was attenuated by ACE. Interestingly, p-4EBP1 was lower with exercise compared to the non-exercise condition, and this response was attenuated by ACE. This change in translational signaling did not affect myosin heavy chain in the soleus or quadriceps, which is consistent with our observation that ACE did not improve treadmill exercise performance. In follow-up experiments to test higher doses of ACE in vitro, we found that ACE had no effects on muscle cell proliferation or myotube size at any dose tested and only reduced myotube fusion at the highest concentrations (200 μM). Taken together, our data suggests that low doses of ACE do not strongly affect the musculoskeletal system alone or when coupled with treadmill running in rodents.

A recent review suggested that NSAIDs has the potential to attenuate or inhibit the osteogenic response to loading.[57] This speculation was based on studies that found indomethacin, NS-398, ibuprofen, and naproxen are detrimental to adaptive bone formation or bone healing.[58–60] The mechanism underpinning these changes is thought to be due to the inhibition of COX-2, which is an inducible COX isoform that is increased after exercise or injury.[60,61] ACE is a selective COX-2 inhibitor but has a much lower affinity for COX-2 than celecoxib.[61] Previous research suggests treadmill running leads to increases in bone formation markers, and bone metabolism and decreases in bone resorption markers, resulting in beneficial osteogenic effects on bone formation.[29,62,63] Our study was specifically designed to build on Liu et al.,[64]

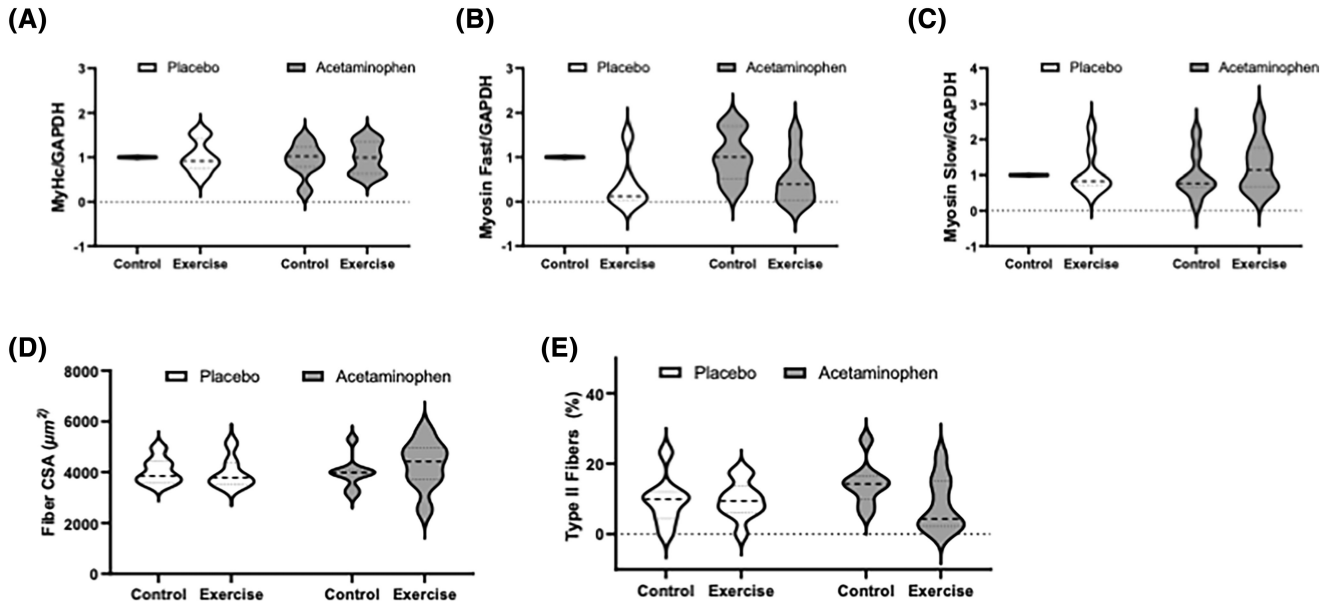

FIGURE 6 Soleus myosin heavy chain content and cross-sectional area. Effects of acetaminophen on myosin heavy chain protein expression and cross-sectional areas in soleus muscle. Western immunoblotting was performed using protein lysates using the following antibodies: (A) myosin heavy chain (MyHc), (B) myosin heavy chain slow, and (C) myosin heavy chain fast. Results were all first normalized to GAPDH and then expressed relative to placebo control which is set to 1.0. Data are representative of $n = 9–10$ and expressed as violin plots. Soleus were sliced using a microtome to an 8 μM thickness and mounted on slides and stained for Type I fibers, Type II fibers, and laminin. (D) Fiber cross sectional area, and (E) Type II fiber percentage were analyzed. Data are representative of $n = 5–7$ per group and expressed as violin plots with medians.

who tested the effects of treadmill running with different speeds (12, 16, or 20 m/min) on bone quality and muscle properties in adult rats. They found an increase in failure load and bone volume in femurs using microCT and three-point bending when rats were exercised at 12 m/min for 30 min per day, 5 days a week for 4 weeks. In this study, skeletal muscle weight was increased in the 16 m/min group, but not in others, indicating potential muscle adaptations. While our treadmill protocol was similar, we only found a decrease in Young's Modulus with exercise. One interpretation when comparing our study to Liu et al. is that transient effects at the lowest speeds (12 m/min) and our speeds (15 m/min) or our use of a longer training protocol may have prevented us from finding the muscle and bone phenotypic changes we expected. Comparatively, 12 weeks of treadmill running has been shown to increase bone structural properties by 5%–15% and increase strength properties by 7%–18%.[65] A recent literature review, published at the midpoint of our experiments, suggested that bone adaptations to treadmill running can be inconsistent and the majority of studies that have used treadmill running do not improve bone mechanical properties.[66] Thus, our study helps provide more information to characterize bone adaptations to treadmill exercise. Furthermore, our findings with ACE agree with observations in human studies on bone adaptations with exercise and low-dose NSAIDs, suggesting that low doses have very little effect on bone strength or volume.[67–70]

In skeletal muscle, we found that ACE attenuated the phosphorylation of AMPK and the dephosphorylation of 4EBP1 in response to exercise. It is well established that endurance-based exercise increases phosphorylation of AMPK at the Thr172 site, which increases glucose uptake.[71,72] AMPK also regulates protein translation through multiple mechanisms related to the mechanistic target of rapamycin, complex 1 (mTORC1) pathway. For example, others have found that AMPK phosphorylation is elevated ~2.5-fold and 4EBP1 phosphorylation is reduced by 60% in mice running on a treadmill for 30 min.[73] A recent review highlighted that phosphorylation of 4EBP1 is reduced immediately after endurance exercise, resulting in a depression of muscle protein synthesis.[74] Additionally, chronic exercise training has no effect on increasing protein synthesis or reducing degradation after prolonged exercise.[75] A few considerations can be drawn from our findings. First, p-4EBP1 during endurance exercise is dependent on intensity and muscle fiber type. Some research indicates that it is predominately elevated in type 2 muscle fibers, and our measurements were taken in the quadriceps, which is a mixed muscle.[76] Second, our data with ACE support previous findings that NSAIDs suppress muscle protein signaling after resistance exercise, but that the

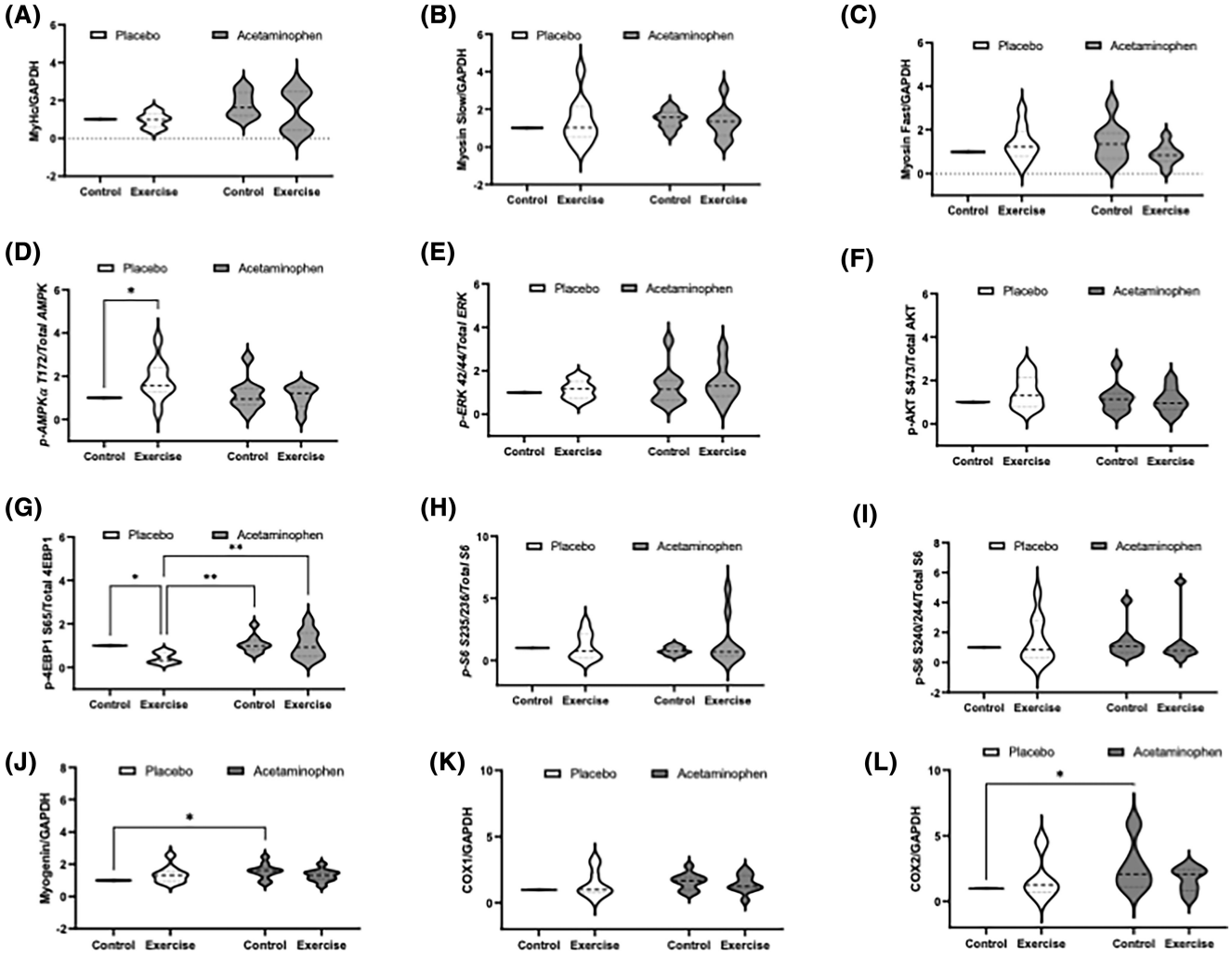

FIGURE 7 Quadriceps Muscle Protein Content. Effects of acetaminophen on myosin heavy chain proteins, anabolic pathway proteins and cyclooxygenase proteins in quadricep muscle. Western immunoblotting was performed using protein lysates using the following antibodies: (A) myosin heavy chain (MyHc), (B) myosin heavy chain slow, (C) myosin heavy chain fast, (D) phospho-AMPKα Thr172, (E) phospho-ERK 42/44, (F) phospho-AKT Ser473, (G) phospho-4EBP1 Ser65, (H) phospho-S6 Ser235/236, (I) phospho-S6 Ser240/244, (J) Myogenin, (K) COX1, and (L) COX2. Results were all first normalized to GAPDH or the corresponding total protein then expressed relative to placebo control which is set to 1.0 Data are representative of $n = 7–8$ per group and expressed as violin plots with medians (*$p < .05$, **$p < .01$, **$p < .001$).

blunted translational signaling did not cause any long-term effects on muscle fiber cross-sectional area or fiber type.[77] When probing the molecular pathways involved in skeletal muscle recovery and adaptations, we found no changes in transcriptional regulation of myosin heavy chain, mef2c, NFATc1, or PGC-1a signaling post-exercise, which indicates there are minimal acute signaling alterations in our experiments. Under non-exercise conditions, we found a small increase in myogenin protein content with ACE, which plays a role in the satellite cell response, and is attenuated by NSAIDs in some studies[78] and stimulated in others.[79]

To better understand the high-concentration effects of ACE in skeletal muscle, we used a human muscle cell culture model similar to previous experiments.[42,43] For this, we tested a range of doses of ACE that replicate concentrations in human blood and extend far beyond it, with the primary goal of determining if muscle cell proliferation, fusion, or myotube size would be affected. Interestingly, our results indicated that only ACE at the highest concentrations had a negative effect, resulting in reduced fusion index, which is like previous findings with arachidonic acid.[42] Furthermore, in experiments testing the effects of celecoxib and NS-398, two strong COX-2 inhibitors, we found that celecoxib negatively reduces myoblast proliferation and differentiation through unknown mechanisms independent of COX-2 inhibition.[51] Cumulatively, our data, in combination

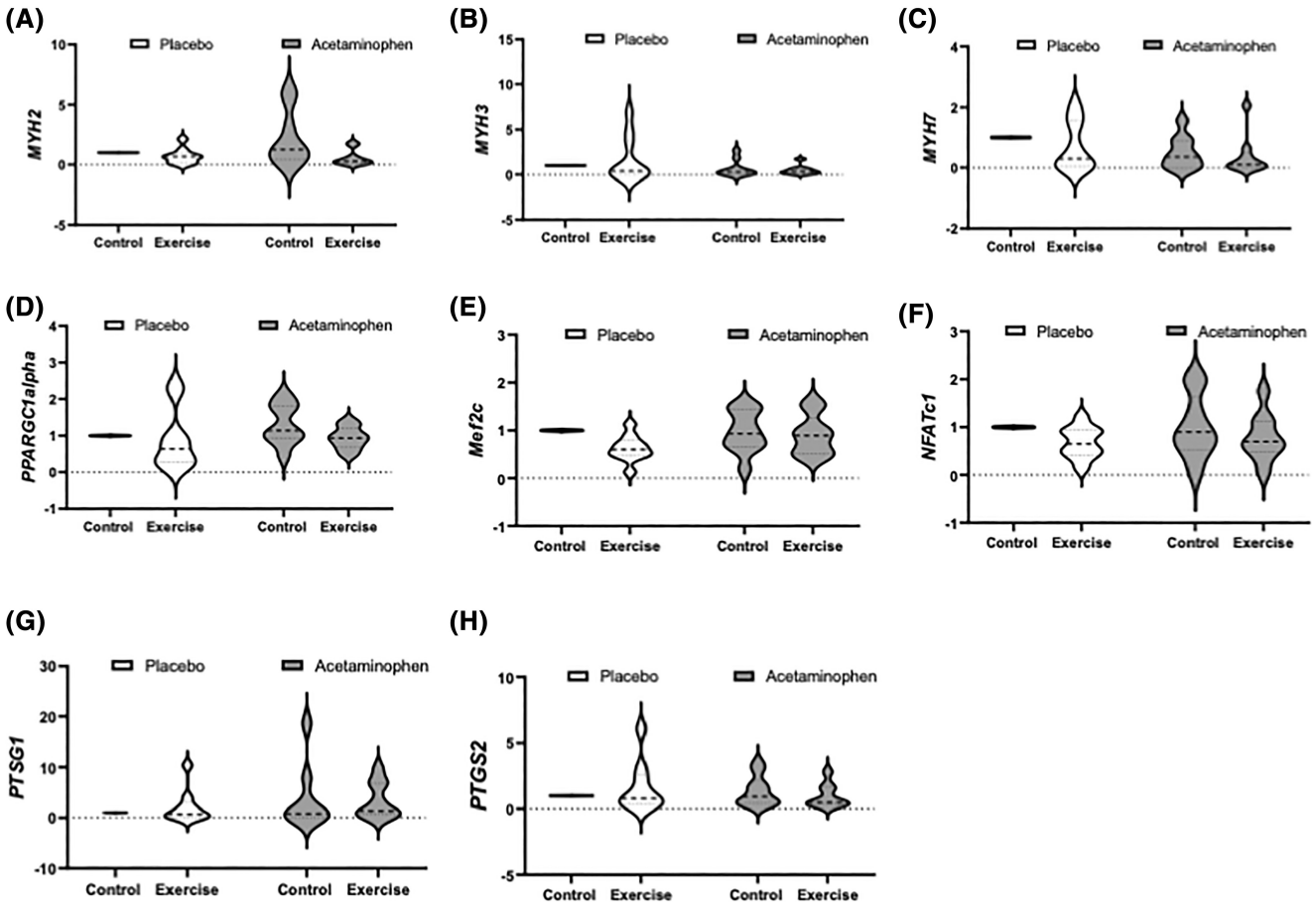

FIGURE 8 Quadriceps Muscle mRNA Expression. Effects of acetaminophen on muscle mRNA expression in Quadricep muscle. qRT-PCR was performed on cDNA derived from each animal to measure the abundance of each of the following genes: (A) MYH2 (B) MYH3, and (C) MYH7, (D) PPARGC1A, (E) MEF2C, (F) NFATC1, (G) PTGS2, and (H) PTGS1. Results were first normalized to 18S then expressed relative to placebo control, which is set to 1.0. Data are representative of $n = 7–8$ per group and expressed as violin plots with medians ($^*p < .05$).

with previous research, indicate that ACE might be a better alternative to traditional NSAIDs in some situations since it was not detrimental to skeletal muscle and had minimal effects on bone.

Our study has several strengths. First, we quantified aspects often ignored in animal research such as body weight, food, and water intake. Second, we measured multiple muscle types, identifying changes in the soleus and quadriceps. Third, we comprehensively analyzed phenotypic properties of bone and skeletal muscle. However, our study also has some limitations. We used a low dose of ACE, which may not be relevant for those consuming high doses post-injury. We also gave ACE diluted in the drinking water, which is like previous work,[80,81] but results may have differed if given via other routes (e.g., oral gavage, intraperitoneal injection, etc.). Using back-calculations based on rats water intake (47.3–49.2 mL/day) across the study our dosage translated to ~45 mg/kg/day of ACE, which is above what Wu et al., used.[17] We also did not measure serum ACE levels, but ours are likely below what other studies have found.[82,83] Furthermore, rats drank ad libitum and thus we did not control the timing of ACE consumption, which may play a role in musculoskeletal signaling that was measured.[67]

Future research is warranted on the effects of higher dosages of ACE (i.e., equivalent to prescribed dosages given for pain management in response to severe injury) to see if the findings of the current work at lower dosages hold true. Moreover, studies looking at further cellular mechanisms in response to ACE consumption and exercise training, such as the effects on mitochondria and stress response pathways to exercise, should also be investigated. Other tissues, such as tendons and cardiac muscle, should also be studied given the negative effects of ACE found by others.[82–85] Finally, follow-up studies examining the influence of various dosages of ACE while undergoing concurrent training (i.e., both aerobic and resistance training) on musculoskeletal adaptations to exercise is also warranted given recent findings with

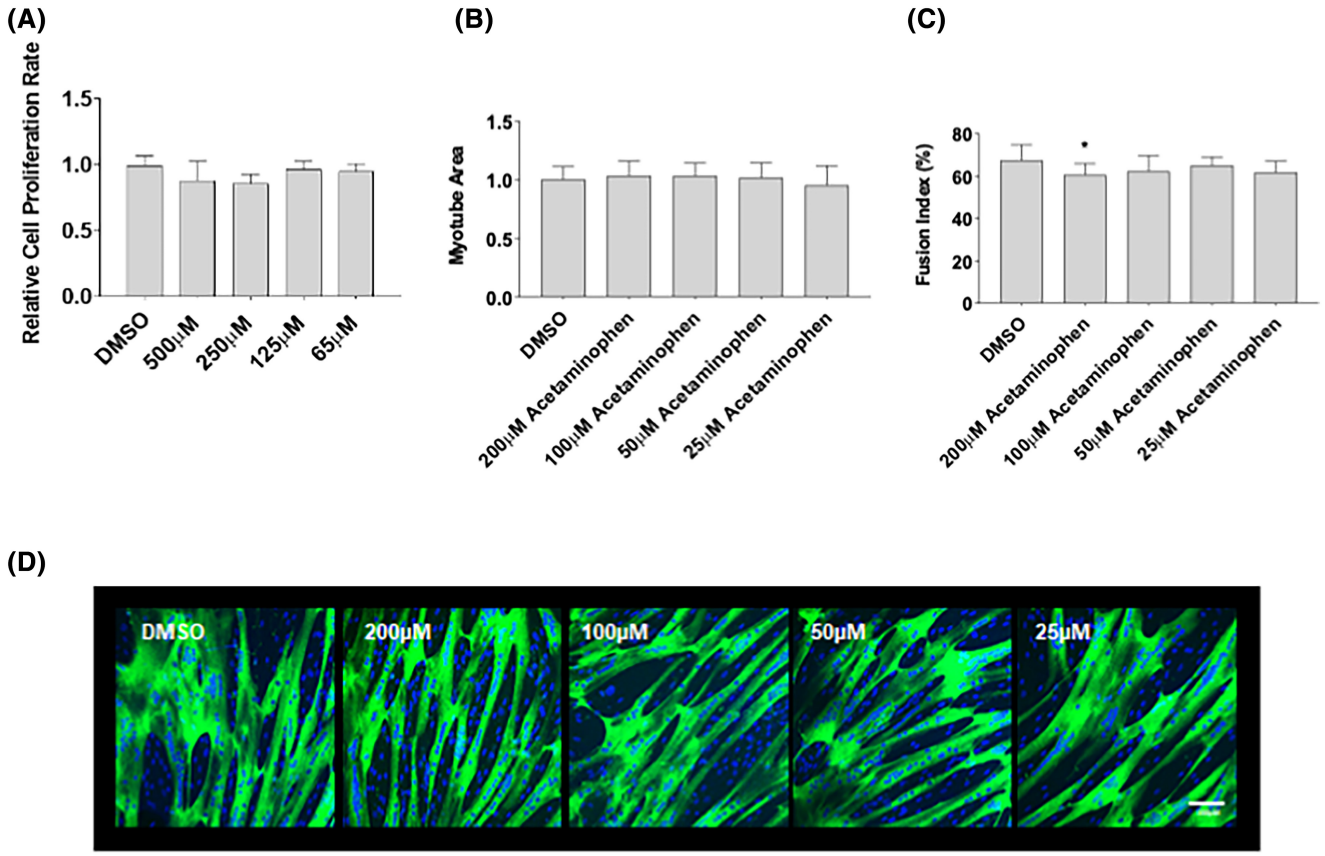

FIGURE 9 In vitro experiments. Effects of Acetaminophen on myotube proliferation and differentiation. Myoblasts were proliferated for 48 h while treated with vehicle (DMSO) or various doses of acetaminophen and (A) proliferation rate was measure by MTT assay. Data are representative of four induvial experiments and expressed as mean ± *SD*. Myotubes were differentiated for 72 h while treated with vehicle (DMSO) or various doses of acetaminophen and (B) myotube area and (C) fusion index was measured. (D) Images were obtained using a Zeiss LSM confocal microscope. Data are representative of three individual experiments and expressed as mean ± *SD* (*$p < .05$).

NSAIDs.[86] In conclusion, our findings indicate that a low dose of ACE, which was equivalent to ~400 mg of ACE per day for a human, had a minimal affect on musculoskeletal adaptations in rodents who were exercising for 6 weeks.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to data sharing agreements required by the author's organization (USARIEM).

DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. Supported in part by an appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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