Serial Measurements of Systemic Myokine Levels in Exercised Foals: A Pilot Study

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INTRODUCTION: Catastrophic musculoskeletal injuries in the appendicular skeleton of horses are a significant welfare concern in horses and are primarily a result of fatigue loading.[1,2] Bone (re)modeling during growth and in response to exercise is a complex process, and a better understanding of the factors that influence remodeling could lead to more effective strategies to prevent fractures. It is well known that the contraction of skeletal muscle during locomotion stimulates bone by exerting forces, but muscle contraction may also provide a biochemical stimulus for bone modeling/remodeling via the expression of myokines[3]. Myokines could act in a paracrine or endocrine fashion. The myokines RANK-L, irisin, and MMP-2 are known to be important components of canonical bone remodeling pathways and are influenced by age. Cross-sectional studies in humans have shown that RANK-L increases with age during skeletal growth[6,7] and MMP-2 also increased with age in an elderly population[4]. In contrast, irisin levels decrease with age during both pre- and post-skeletal maturity[5]. However, the effect of exercise on myokine expression remains unclear with conflicting reports in the literature[8,9,10,11,12], and there have been no reports of the effect of a multi-week exercise program on circulating myokine levels during growth. Moreover, the degree to which myokine expression changes longitudinally within an individual is not clear. Therefore, the objective of this study was to investigate the effects of both age and exercise on systemic myokine levels in growing foals using serial measurements.

METHODS: All procedures were approved by the University of Illinois Urbana-Champaign IACUC (protocol #21136). **Cohort and exercise intervention:** Six foals underwent an 8-week exercise protocol starting at two months of age. Six age-matched controls were not exercised. The exercise regimen consisted of leading the dam while the foal followed: 1200m for the first week and then 1600m for seven weeks (5 days/week) at an average speed of 3.5 m/sec. All foals and dams were housed in the same pasture for the duration of the intervention period. **Blood collection:** Blood was collected from the jugular vein of all foals (control and exercise) at 8, 10, 12, 16, and 20 weeks of age, and 8 months and 11 months of age. Blood samples were spun down, and the serum was separated and stored at -80°C until analysis. **Myokine measurement:** Myokine concentrations in serum were measured by commercially available sandwich ELISAs that had horse-specific antibodies to RANK-L, irisin, and MMP-2, according to manufacturer instruction. **Data processing:** Standard curve equations for each ELISA were fitted using a 4PL regression model (mycurvefit.com) and all analyses were performed using R. Data were tested for normality using a Shapiro-Wilk test. Given that the data were not normal, a Friedman test and Wilcoxon-Signed Rank Test were used to determine if there was a difference in concentration between time points, while Mann-Whitney-U tests were used to compare exercise versus control foals at each measured timepoint. Data are presented as median [interquartile range]. Significance was set at $p < 0.05$ with Bonferroni correction applied as appropriate.

RESULTS: Overall, there were changes in systemic myokine levels with age but not between exercised and non-exercised groups. There was a small increase in RANK-L ($p=0.01$) with age, with a significant difference between 8 weeks (24.7pg/mL [21.6-33.6pg/mL]) and 18 weeks (34.0pg/mL [28.2-36.1pg/mL]; $p=0.03$). In contrast, the systemic levels of irisin decreased ($p<0.0001$) consistently throughout the study period. At 8 weeks of age, the median irisin measurement was 145.2ng/mL [57.3-197.3ng/mL] compared to only 11.9ng/mL [9.4-19.2ng/mL] at 11 months of age ($p=0.001$). There was an increase in MMP-2 levels with age ($p=0.004$); however, pairwise comparisons between timepoints were not statistically significant. The difference between 10 weeks (33.3pg/mL [23.5-54.6pg/mL]) and 16 weeks approached significance (77.7pg/mL [61.3-139.1pg/mL]; $p=0.07$). There was no difference in systemic RANK-L, irisin, or MMP-2 levels at any time point between foals that underwent the 8-week exercise protocol and control foals.

DISCUSSION: Our findings of increased RANK-L and decreased irisin expression with age using serial measurements agree with the previous cross-sectional data reported in humans. RANK-L is a key regulator of bone resorption through its role as a stimulator of osteoclast activity. The increase in RANK-L may be due to the rapid bone growth in foals during the first year of life which includes endosteal resorption and the modification of plexiform to Haversian bone. Irisin is associated with increased osteoblast activity, differentiation, and proliferation,[13] which would then be expected to increase due to bone formation. However, we found a systemic decrease in irisin similar to other studies in growing humans. Irisin is also implicated in the browning of fat[14] and metabolism. Young animals have larger stores of brown fat that decrease with age and this may be responsible for the decrease in circulating irisin over the course of the study period. Finally, circulating levels of MMP-2 have not previously been reported in a young cohort in any species. However, as MMP-2 plays an important role in normal endochondral ossification, this may explain why this myokine tended to increase with age during a period of rapid growth. Replication of these findings in a larger study cohort is needed.

When considering why there was no change in myokine levels with exercise, it is possible that the exercise program was not intense enough or long enough to result in sustained changes in systemic myokine levels beyond that already occurring during normal growth. Current work includes evaluation of changes in bone properties based on computed tomography data to assess the effect of the exercise intervention on bone. It is also likely that our sampling scheme, in which samples were collected either before or several hours after daily exercise, was not appropriate to capture transient increases in myokine levels associated with exercise events. Finally, it may be that myokines exert their effects on bone in a paracrine rather than endocrine fashion and that local changes in expression in the muscles rather than systemic levels more accurately reflect their activity; this is the subject of ongoing work. Future studies with more intense exercise conditions, more subjects, and additional serial measurements are needed to confirm these results.

SIGNIFICANCE/CLINICAL RELEVANCE: This work is the first to document serial measurements of systemic myokines in the same subjects throughout the first year of life. Establishing normal levels of myokines and normal changes with age has important implications for better discerning the mechanical and biochemical role of muscles on bone during normal bone development as well as in response to exercise.

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