Histomorphometric evaluation of the effect of early exercise on subchondral vascularity in the third carpal bone of horses

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Objective—To investigate histomorphometric changes in the cartilage and subchondral bone of the third carpal bone associated with conditioning exercise in young Thoroughbreds.

Animals—Nine 18-month-old Thoroughbreds.

Procedures—Both third carpal bones of 9 horses (4 exercised spontaneously at pasture only and 5 given additional conditioning exercise beginning at a mean age of 3 weeks) were evaluated. Histomorphometric variables (hyaline and calcified cartilage thickness and collagen orientation; vascular channel area, number, and orientation; and osteochondral junction rugosity) of the third carpal bone, sampled at 4 dorsopalmar sites in the radial facet, were compared between the exercised and nonexercised groups.

Results—The vascular channel area measured at the 4 dorsopalmar sites was larger in the exercised group than in the control group, but none of the variables were significantly different between groups. Both groups had significant site-specific variations in all measured variables. Most importantly, the vascular channel area was highest in the most dorsal aspect.

Conclusions and Clinical Relevance—Results suggested that the mild exercise imposed in both groups during the developmental period appeared to be associated with an increase in the vascular channel area beneath the calcified cartilage layer in the third carpal bone. This increased vascular channel area could also be associated with high stress in the dorsal aspect of the radial facet, a region that is known to be vulnerable to osteochondral fragmentation. (Am J Vet Res 2013;74:542–549)
tional conditioning exercise group; 6). Carpal bones of 3 of these horses were randomly chosen and used in establishing the required experimental procedures; of the remaining 9 horses, 5 were in the additional conditioning exercise group and 4 were in the control group. These 12 horses had been euthanized at 18 months of age, and the tissues were also used in other studies, including the evaluation of the same third carpal bones of the present study. The study and its procedures were approved by the Massey University Animal Ethics Committee.

Exercise program—For approximately 18 months, beginning at a mean age of 3 weeks, both groups of horses were kept on pasture, but horses in the additional conditioning exercise group were additionally exercised (1,030 m/d on 5 d/wk) on an oval track under controlled conditions described elsewhere. During phase 1A (from 3 weeks of age to weaning [approx 120 days]), the mean target speed for exercise was 5.4 m/s; in phase 1B (from weaning to first sprint [approx 100 days]), the mean target speed was 7.5 m/s; and in phase 1C (approx 300 days), the mean target speed was 9.6 m/s, with a brief 129-m sprint at 12.5 m/s. During the 18-month exercise period, prolonged lameness was not detected in any of the 12 horses, whereas during brief periods, mild and moderate effusions in the midcarpal joints were found in an earlier study of the larger cohort of horses, of which the 12 in the present study were part.

Sample collection and processing—From each of the 18 third carpal bones, osteochondral slabs (approx $20 \times 10 \times 5 \text{ mm}$) were sawn immediately adjacent to the location of the slab cut for a study of cartilage swelling (Figure 1). The slabs were divided into sites 1 and 2 (exposed to high but intermittent loading) and sites 3 and 4 (exposed to moderate and more constant loading). As a control, site 0 was chosen from the nonloaded region of the joint surface adjacent to site 1, at the extreme edge of the dorsal lip of the radial facet. The slabs were fixed in 10% formaldehyde solution for 1 to 3 days, decalcified for up to 7 days in 10% formic acid solution buffered with sodium formate, rinsed in running water for 1 hour, and mounted with a formulation of water-soluble glycols and resins on a slide microtome for cryosectioning. From each slab, 30-µm-thick osteochondral serial sections ($n = 10$ to 30) were obtained in the sagittal plane, spanning a distance of approximately 3 mm. Five sections were randomly selected for histologic examination and photographic (bright-field microscopy) imaging. The selected sections were washed with water at room temperature (approx 22°C), wet-mounted under a coverslip on a glass slide for examination at 20X magnification (unstained), and digitally photographed. If the section contained focally enlarged calcified cartilage as described, the affected section was not included in the study.

Figure 1—Illustrations and photograph of the forelimb and third carpal bone of a horse. Left panel—Diagram of the skeletal structures of the forelimb. The third carpal bone is indicated in red; the arrow indicates the orientation reference between A and B. A—Diagram of the third carpal bone indicating the location of histomorphometric examination sites 0 to 4 (asterisk) immediately adjacent to sampling sites previously used to obtain matrix swelling data. Osteochondral lesions are frequently reported in the dorsal region of the facet (red oval). B—Photograph of a portion of the third carpal bone indicating sites 0 to 4 of a typical sagittally sectioned osteochondral slab.

Figure 2—Illustration of an osteochondral slab of the third carpal bone of a horse indicating the histomorphometric variables examined in sections obtained as indicated in Figure 1. Vascular channels are labeled with numerals 1 to 5. CCang = Collagen matrix orientation in calcified cartilage. CCT = Calcified cartilage thickness. HCang = Collagen matrix orientation in hyaline cartilage. H Ct = Hyaline cartilage thickness. Rug = Osteochondral junction rugosity, defined as length of osteochondral junction divided by the grid unit length (3,000 µm). Vca/Vcn = Vascular channel area. VCang = Mean orientation of vascular channels. Vcn = Number of vascular channels.
**Histomorphometric assessment**—Each high-resolution composite image of the osteochondral section was overlaid with four 3,000 × 3,000-µm square grids covering the matrix beneath the en face sites 1 to 4 (Figures 2 and 3). Within the grid, a pen-tablet digitizer with image editing software was used to trace by hand the relevant tissue boundaries, which were then digitally colored (blue for hyaline cartilage, green for calcified cartilage, and red for vascular channels). Because vascular channels are thought to participate in remodeling of the osteochondral junction via advancement up to and into the calcified cartilage,27–30 they were counted only if they made contact with the osteochondral junction or were within approximately 50 µm of it. The area of any vascular channel intruding into the calcified cartilage was also included.

Each grid square was analyzed with image analysis software to quantify the tissue variables, namely thickness of hyaline cartilage and calcified cartilage and area and number of vascular channels. A previous study revealed that the alignment of the chondrocytes provides a clear indication of the overall fibrillar alignment. By use of this method, the general fibril orientations in the hyaline cartilage and calcified cartilage were measured from a reference line drawn perpendicular to the articular surface by determining the mean of 10 measurements. The orientation of the vascular channels was calculated by determining the mean of all those previously selected. The rugosity of the osteochondral junction was measured by dividing the length of the osteochondral junction by the grid length (3,000 µm).

**Statistical analysis**—Five osteochondral sections per bone from 18 bones (left and right) of 9 horses resulted in 90 osteochondral sections. This, in turn, generated 360 data sets (ie, 90 sections × 4 sites), and each data set consisted of the 8 histomorphometric variables (hyaline cartilage thickness, calcified cartilage thickness, vascular channel area, number of vascular channels, fibril orientations in the hyaline cartilage, fibril orientations in the calcified cartilage, orientation of the vascular channels, and osteochondral junction rugosity). Thirty-five data sets of the 360 histologic slices from sites 1 or

![Diagram](image_url)
affected with calcified cartilage abnormality (abnormally thickened calcified cartilage and disrupted osteochondral junction) were excluded from the assessment. This exclusion did not affect the overall significance ($P = 0.53$) of the treatment effect as verified by use of an exact $\chi^2$ significance test.

The remaining 325 data sets were analyzed with a statistical software package via both multivariate ANOVA for a broad assessment of the exercise effects and univariate mixed model analyses (ANOVA) on each of the histomorphometric variables for variable-specific treatment effects. The model included nested random effects of horse and site, and dependence of the left and right leg was also factored into the models. Values of $P < 0.05$ were considered significant.

Ten correlation coefficients ($R$) between each pair of the 5 nonangular histomorphometric variables (ie, hyaline cartilage thickness, calcified cartilage thickness, vascular channel area, number of vascular channels, and osteochondral junction rugosity) were calculated and used to construct correlation matrices for each group. Ten thousand permutation tests incorporating Fisher $Z$ transformation were performed to determine

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exercised</th>
<th>Control</th>
<th>$\Delta R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCt-CCt</td>
<td>0.59</td>
<td>&lt; 0.001</td>
<td>0.08</td>
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<tr>
<td>HCt-VCa</td>
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<td>0.015</td>
<td>0.07</td>
</tr>
<tr>
<td>HCt-VCn</td>
<td>0.34</td>
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<tr>
<td>HCt-Rug</td>
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<td>0.004</td>
<td>0.38</td>
</tr>
<tr>
<td>CCt-VCa</td>
<td>0.36</td>
<td>&lt; 0.001</td>
<td>0.27</td>
</tr>
<tr>
<td>CCt-VCn</td>
<td>0.42</td>
<td>&lt; 0.001</td>
<td>0.34</td>
</tr>
<tr>
<td>CCt-Rug</td>
<td>0.30</td>
<td>&lt; 0.001</td>
<td>0.33</td>
</tr>
<tr>
<td>VCn-Rug</td>
<td>0.42</td>
<td>&lt; 0.001</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Only those pairs with values of $P < 0.05$ are listed.

*Notice that the correlation coefficient of HCt-CCt in the exercised group is much higher than that for the control ($\Delta R = 0.50$) suggesting that there was a measurable exercise effect in terms of the HCt-CCt relationship.

CCang = Collagen matrix orientation in calcified cartilage. CCt = Calcified cartilage thickness. HCang = Collagen matrix orientation in hyaline cartilage. HCt = Hyaline cartilage thickness. Rug = Osteochondral junction rugosity, defined as length of osteochondral junction divided by the grid unit length (3,000 µm). VCa = Vascular channel area. VCang = Mean orientation of vascular channels. VCn = Number of vascular channels.
whether there was any evidence of structural difference between the matrices.

**Results**

An overall exercise effect encompassing all the histomorphometric variables was not detectable \( (P = 0.396; \text{Figure 4}) \). When each variable was considered individually, there were measurable differences in the vascular channel area between the groups across all sites 1 to 4; the mean values of the additional conditioning exercise groups’ vascular channel area were consistently (33% to 57%) higher than those of the control group, but this difference was not significant \( (P = 0.057) \) because of large variance in the data. The 7 other variables had neither differences in their mean values nor significant detectable exercise effects.

Both groups had strong site-specific findings in all measured histomorphometric variables regardless of the exercise treatment \( (P < 0.001) \). Although the largest vascular channel area occurred at site 1, this decreased progressively toward the palmar aspect. All the other nonangular variables (hyaline cartilage thickness, calcified cartilage thickness, number of vascular channels, and osteochondral junction rugosity) had maximums at sites 2 or 3 and minimums at either site 1 or 4. The variables fibril orientations in the hyaline cartilage, fibril orientations in the calcified cartilage, and orientation of the vascular channels all had a consistent increase from site 1 to 4 (range, −40° to 20°), with neutral orientations (ie, perpendicular to the articular surface) prevailing in sites 2 and 3.

The Pearson correlation coefficients \( (R) \) for the additional conditioning exercise group and control group indicated that most of the additional conditioning exercise group \( R \) values were larger than those of the control group by up to 0.5 (Table 1). However, when tested for the difference between within-group correlation matrix structures by use of the permutation tests, no significant \( (P = 0.063) \) difference was detected between the groups.

**Discussion**

This study measured and compared 8 histomorphometric variables relating to the hyaline and calcified cartilage and the vascular channels in the radial facet of the third carpal bones of the additional conditioning exercise group and the control group. Although the study failed to detect a significant exercise effect, the mean vascular channel area of the additional conditioning exercise group was larger by 35% to 57% in all measured sites, compared with that of the control group. Furthermore, vascular channel area of both groups had the highest values at site 1 (ie, the most dorsal region of radial facet in the third carpal bone), whereas the remaining variables had highest values at site 2 or 3 (ie, midpoint of radial facet).

Vascular channels are the fine osseous cavities (diameter, 10 to 30 µm) formed by ongoing osteoclastic resorption as a part of bone remodeling via the activation-resorption-formation sequence.\(^{32}\) Although the function of the vascular channels is not fully understood, they are thought to be important features in bone, associated with nutritional and signal pathways into the overlying hyaline cartilage\(^{33–35}\) as well as with cartilage mineralization and tidemark advancement.\(^{36,37}\)

The detailed quantification of vascular channel area enlargement performed in this study is, to the authors’ knowledge, the first of its kind. The findings are supported by others who have also reported positive associations between the vascular channel area and exercise in the femoral head\(^{38}\) and tibial diaphysis\(^{39,40}\) of mice. Thus, greater vascular channel area in the additional conditioning exercise group and in the highly to intermittently loaded site 1 of the third carpal bone\(^{41–43}\) in both groups (additional conditioning exercise group and control group) does suggest that subchondral vascular channel enlargement could be an adaptive response of the subchondral bone to increased stress. However, the mechanism of vascular channel enlargement is not understood well; it could be driven by the need to repair microfractures associated with stress\(^{44–46}\) or by the increased blood flow (hyperemia) from the cyclic loading of the bone.\(^{47}\)

Although vascular channel enlargement is likely to be a normal adaptive feature in the subchondral bone, the pronounced enlargement observed at site 1 is of potential concern in that such a structural change could compromise the mechanical strength of the third carpal bone’s subchondral bone. An increase in both vascular channel area and vascular channel density has been reported to weaken human cortical bone by reducing its fracture toughness\(^{48}\) and fatigue strength,\(^{49}\) respectively. Similarly, the greater vascular channel enlargements in the exercised group in the present study could reduce the bone’s structure integrity, thus lowering the strength of the third carpal bone in a repetitive stress environment. This may explain why authors of the present study have also observed osteochondral abnormalities characterized by calcified cartilage thickening and a disruption of the cement line, together with a variety of other unusual structural features in the dorsal region of the third carpal and the opposing radial carpal bone.\(^{19}\)

Thus, the observation of vascular channel enlargement suggests that further investigation is required to establish with greater statistical certainty that it does have a true association with exercise. This is especially so given that dorsal site 1 is the region often associated with osteochondral fracture, subchondral bone collapse, and osteoarthritis.\(^{10}\)

All 8 histomorphometric variables in both groups had strong site-specific characteristics. A similar site-specific pattern in the swelling behavior of the hyaline cartilage was found in another study\(^{40}\) of the same bones, suggesting that there is an important influence of the pattern of loading across the third carpal bone surface irrespective of the exercise treatment.

With regard to hyaline cartilage thickness and calcified cartilage thickness, their site specificities are thought to arise from localized adaptations that increase joint congruency,\(^{50–52}\) this in turn being driven by the contact stresses generated by weight bearing and joint movement.\(^{53–55}\) Although other studies that used equine tissues have also found an overall positive correlation between hyaline cartilage thickness and added exercise in the metacarpal condyle,\(^{18}\) carpus,\(^{14,36}\) and
Their angular morphology might be a consequence of lowed closely the site-specific patterns in fibril orien-
tality, compared with sites 1 and 4. The carpal bone, did not have any strong collagen direction-
has little or no contact stress from the opposite radial
terestingly, the extreme dorsal edge (ie, site 0), which
generated while embedding the deviated
collagen fibrils, this may result in an overall fibrillar deviation
from the advancing vascular channels; the irregular ce-
cement line associated with this advance is thought to fa-
cilitate extra anchorage between the cartilage and sub-
chondral bone. Although other studies have found the
degree of interdigitation to be positively correlated both with age and stress on the bone, the present study did not find any significant variation among the 4 sites where the values ranged from 1.8 to 1.9. However, the pattern of variation resembled that of the number of vascular channels, with the correlation coefficients between osteochondral junction rugosity and number of vascular channels being 0.42 and 0.35 for the additional conditioning exercise group and control group, respectively. These values suggest that there could be a positive correlation between osteochondral junction rugosity and the number of vascular channels.

Collagen fibril orientation and organization are important factors influencing the mechanical function of articular cartilage. In the present study, semi-quantitative measurement of collagen fibril orientation in the deep zone of the hyaline cartilage and calcified cartilage indicated that the collagen fibrils have strong site-specific angular deviations from the primary radial direction (ie, perpendicular to the articular surface; Figure 3). Such deviations may be an adaptive response to accommodate localized loading during the growth phase. We suggest that the collagen fibrils in the dorsal and palmar aspects had undergone lateral spreading because of a combination of axial compression and local shear forces. With the deep anchorage of the collagen fibrils, this may result in an overall fibrillar deviation away from the radial direction. As the joint matures, the deep zone matrix, in its laterally sheared state, is then progressively calcified while embedding the deviated fibrils, as is indicated by the chondrocyte flow lines. Interestingly, the extreme dorsal edge (ie, site 0), which has little or no contact stress from the opposite radial carpal bone, did not have any strong collagen directionality, compared with sites 1 and 4.

Even the orientation of the vascular channels followed closely the site-specific patterns in fibril orientations in the hyaline cartilage and calcified cartilage. Their angular morphology might be a consequence of a developmental adaptation influenced by the loading pattern across the joint surface during the horse's early growth phase. Interestingly, the results appear similar, if not identical, to the changes observed (via different methodologies) in the cartilage of the proximal phalanx of these same horses at the same age.

This study used histomorphometry to reveal that vascular channel morphology was influenced by both early exercise treatment and regional stress patterns occurring in the radial facet of the third carpal bone. Results suggested that the mild exercise imposed during the developmental period appeared to be associated with an increase in the vascular channel area beneath the calcified cartilage layer in the dorsal aspect of the bone, although the difference from controls was not significant. The potential for such changes to compromise the strength and fracture toughness of the third carpal bone is of particular concern in view of its known vulnerability to osteochondral fracture and related joint disease.

References


