Evaluation of age-related changes in the structure of the equine tarsometatarsal osteochondral unit

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Objective—To investigate effects of age on thickness and morphologic characteristics of hyaline cartilage, calcified cartilage, total cartilage, and subchondral bone (SCB) in the equine tarsometatarsal joint.

Sample Population—23 tarsal joints from cadavers of 23 ponies (11 days to 25 years old); ponies were limited to pasture exercise and euthanatized for reasons not related to this study.

Procedures—Tarsi were allocated into several age groups (11 days old [n = 3], 6 to 9 months old [4], 2 to 3 years old [3], 6 to 8 years old [4], 11 to 17 years old [6], and 20 to 25 years old [3]). Histologic examination and histomorphometric measurement of hyaline cartilage, calcified cartilage, total cartilage, and SCB were performed at medial and lateral sites.

Results—A significant decrease was detected in thickness of hyaline cartilage and total cartilage with increasing age, but there was a significant increase in thickness of calcified cartilage and SCB with increasing age. Differences in chondrocyte and collagen fiber arrangement, tidemark, and osteochondral junction morphology were evident among age groups.

Conclusions and Clinical Relevance—These findings suggested that the various tissues of the osteochondral unit change in different ways with age. The response of each tissue may be related to relative response of the tissues to strains induced by pasture exercise but could have an influence on how the overall properties of the osteochondral unit change with age. The findings may also be suggestive of changes that develop prior to the onset of osteoarthritis. (*Am J Vet Res* 2009;70:30–36)

The osteochondral unit changes with age, and this may be related to the loading environment[1,2]; however, there is limited understanding of the exact changes and the way in which the various tissues interact in the overall adaptive response. Risk of osteoarthritis in humans increases with age, but the way in which aging is associated with the response of joints to loading and development of pathologic changes is not clearly understood.[3,4] It is important to define normal osteochondral changes with age to understand pathologic changes.

Equine joints provide a good example for study. They are loaded soon after birth, the pattern of loading remains similar throughout life, and their size makes it possible to clearly define structural changes in the osteochondral unit. Horses are long-lived and active animals with naturally occurring osteoarthritis,[5] which makes them useful for the study of joint adaptation and degeneration in humans. There is also potential to select material from horses with a known exercise history to account for different loading environments.

Abbreviation

SCB Subchondral bone

The 2 main functions of a synovial joint are to enable movement and to transfer load.[6] Articular cartilage is fundamental to joint function. The layered appearance of cartilage becomes apparent as joint pressure increases during postnatal activity and is associated with depth-dependent variations in the mechanical environment.[7] Composition and structural properties of cartilage change with age.[8] Changes such as fibrillation of the articular surface, a decrease in size and aggregation of proteoglycan aggrecans, and loss of tensile strength may be caused by a decrease in the ability of chondrocytes to maintain and repair the matrix.[4] Age-related degenerative changes of articular cartilage begin early after birth, which affects unloaded areas of cartilage. It

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has been suggested[8] that unloaded cartilage atrophies in the same way as unloaded bone and muscle.

Articular cartilage and SCB work together to transmit load through a joint.[9] Articular cartilage is affected by the mechanical properties of adjacent SCB.[10] It has been reported[11] that changes in SCB, including increases in bone volume and trabecular thickness and decreases in trabecular number and separation, are correlated with increased degeneration of articular cartilage degeneration in the knees of humans. In the equine carpus, it was found that lesions in the articular cartilage often appear adjacent to bone sclerosis.[12] The results of those studies indicate that the relationship between articular cartilage and SCB is important for normal joint function; therefore, it is essential to understand the ways in which both tissues are altered during loading and how changes in one affect the other.

Increased size and density of chondrocytes have been related to increased load.[13] Therefore, chondrocyte structure and distribution may be related to the pattern of load across a joint. Results of other experiments also suggest that chondrocyte arrangement is associated with loading[14] because chondrocyte clusters were detected at dorsal sites, which were associated with high load in the equine carpus. Chondrocyte clustering is also associated with early osteoarthritic changes.[15]

The objective of the study reported here was to use histologic examination to investigate age-related changes in thickness and morphologic characteristics of hyaline cartilage, calcified cartilage, total cartilage, and SCB at specific locations in the tarsometatarsal joint of equids with a history of consistent exercise. It was hypothesized that cartilage morphology would be altered with increasing age, total cartilage thickness would decrease with increasing age, thickness of hyaline cartilage would decrease with increasing age, thickness of calcified cartilage would increase with increasing age, and thickness of SCB would increase with increasing age.

Materials and Methods

Sample population—Twenty-three tarsal joints were collected from cadavers of 23 Welsh ponies. Ponies were 11 days to 25 years old, had been limited solely to pasture exercise throughout their lives, and had no evidence of lameness. Tarsi were allocated into several age groups (11 days old [n = 3], 6 to 9 months old [4], 2 to 3 years old [3], 6 to 8 years old [4], 11 to 17 years old [6], and 20 to 25 years old [3]). All specimens were collected from ponies euthanatized for reasons unrelated to this study. Tarsi were collected within 6 hours after ponies were euthanatized; specimens were stored frozen at –20°C.

Histologic preparation—Frozen tarsi were clamped; a heavy-duty vice was used to grip the distal portion of the tibia and the calcaneus. A large band saw[a] was used to detach the tarsal joints by cutting in a transverse plane proximal to the talocalcaneal-centroquartal joint (proximal intertarsal joint). Bones of the centrodistal and tarsometatarsal joints were cut into sagittal slices (thickness of 4 to 5 mm). To define medial and lateral sites on each tarsus for sectioning, sites previously defined on sagittal 3-dimensional T1-weighted spoiled gradient-echo magnetic resonance images were translated onto the frozen tarsus, as described elsewhere[16,17] (**Figure 1**). A small band saw[b] was used to cut the chosen medial and lateral slices in a transverse and then a dorsal plane to yield dorsal sections through the tarsometatarsal joint that were half the dorsoplantar depth of the third tarsal bone.

Sections were fixed separately in labeled containers by immersion in neutral-buffered 10% formalin for 10 days. When the sections were removed from the formalin, they were washed to remove bone dust and then placed in a rapid decalcifier solution[c] for 2 days. After removal from the rapid decalcifier solution, sections were placed under a stream of tap water for 1 day. Sections then were dehydrated in 70%, 90%, and 3 changes of 100% alcohol and then 3 changes of xylene and 3 changes of molten histologic wax[d] on an automatic tissue processor.[e] Finally, the sections were embedded in blocks of paraffin.

A heavy-duty rotary microtome[f] was used to cut sections (6 µm thick), which were then mounted on gelatin-coated slides created by dipping cleaned

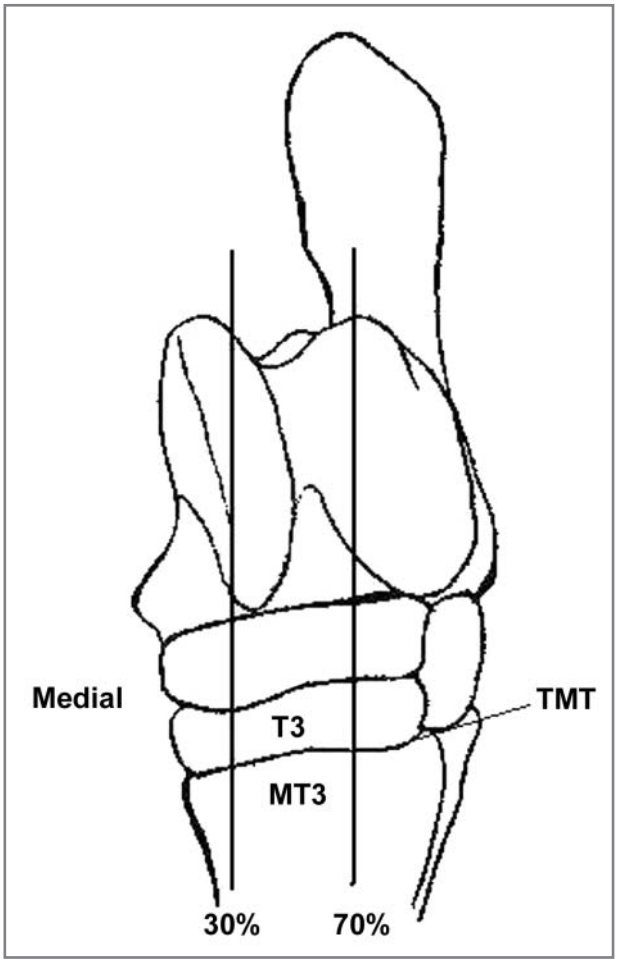

Figure 1—Diagram of the dorsal view of an equine tarsus. The tarsometatarsal joint (TMT) forms the articulation between the third tarsal bone (T3) and third metatarsal bone (MT3). Notice the medial and lateral sites (lines at 30% and 70% of the width of the third tarsal bone, respectively, measured from the medial aspect) where specimens were obtained for histologic examination.

slides in hot gelatin,[g] which were then dried in an oven at 37°C. One slide was stained with Harris' H&E stain, and a second slide was stained with 1% toluidine blue. A coverslip was placed over the stained sections, which were then mounted in mounting medium.[h]

Cartilage morphology was examined by use of a microscope[i] with a linear polarizing light facility. Sections were analyzed to detect defects in the articular surface and osteochondral junction, focal abnormalities in the cartilage and SCB, abnormalities in the ligament structure, erosion of cartilage, formation of osteophytes, abnormally increased areas of dense bone, and focal abnormalities of cancellous bone.

Measurements of the thickness of hyaline cartilage, calcified cartilage, total cartilage, and SCB were obtained from standard sites at dorsal locations (15% of the dorsal-to-plantar dimension of the third tarsal bone) and at medial and lateral sites (30% and 70% of the width of the third tarsal bone, respectively, measured from the medial aspect). In accordance with a validated technique,[18] measurements were obtained from the proximal and distal articular surfaces of the tarsometatarsal joint on digital images of the histologic sections by use of a digital image analysis program.[j] The proportion of hyaline cartilage and of calcified cartilage was calculated as a percentage of the total cartilage thickness at each site.

Data analysis—Morphologic characteristics of articular cartilage in each age group were described and recorded. A Spearman rank correlation was used to test for associations between age and thickness of hyaline cartilage, calcified cartilage, total articular cartilage, or SCB and between age and percentage of total cartilage thickness occupied by the hyaline cartilage and calcified cartilage. Statistical analysis of the results was performed with statistical analysis software.[k] Significance was defined as values of $P < 0.05$.

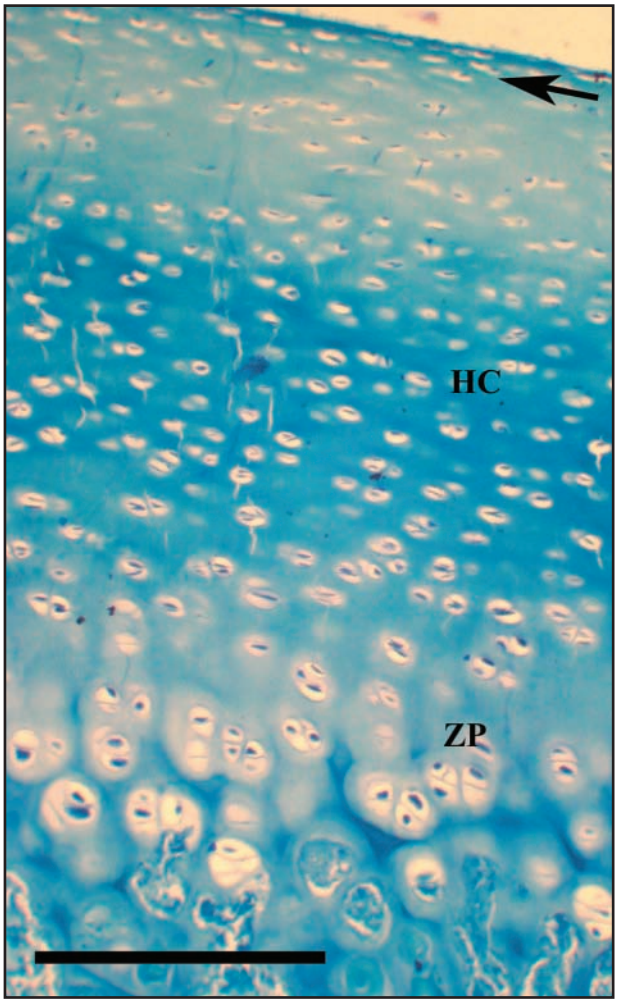

Figure 2—Photomicrograph of a tissue section obtained from the articular surface of the proximal aspect of the third metatarsal bone of an 11-day-old pony. There is thick articular cartilage comprising a thin horizontal layer (arrow), a thick layer of hyaline cartilage (HC), and an active zone of cartilage proliferation (ZP), but there is no distinct calcified zone. Toluidine blue stain; bar = 200 μm.

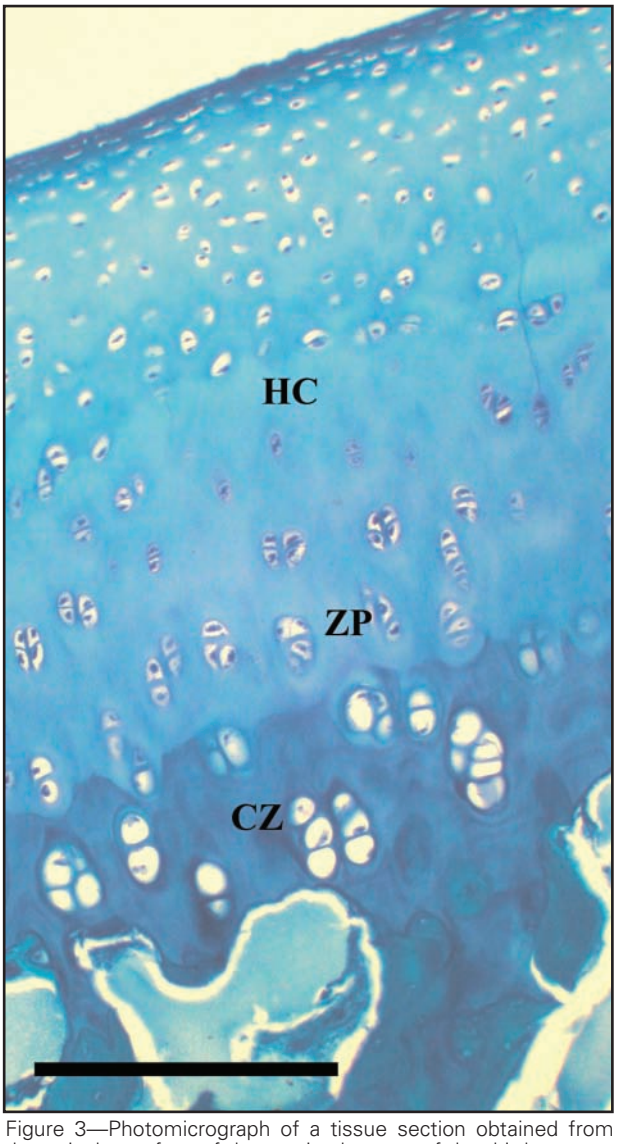

Figure 3—Photomicrograph of a tissue section obtained from the articular surface of the proximal aspect of the third metatarsal bone of an 8-month-old pony. The HC remains thick, but the calcified zone (CZ) is becoming a distinct layer. An active ZP is still evident. Toluidine blue stain; bar = 500 μm. *See* Figure 2 for remainder of key.

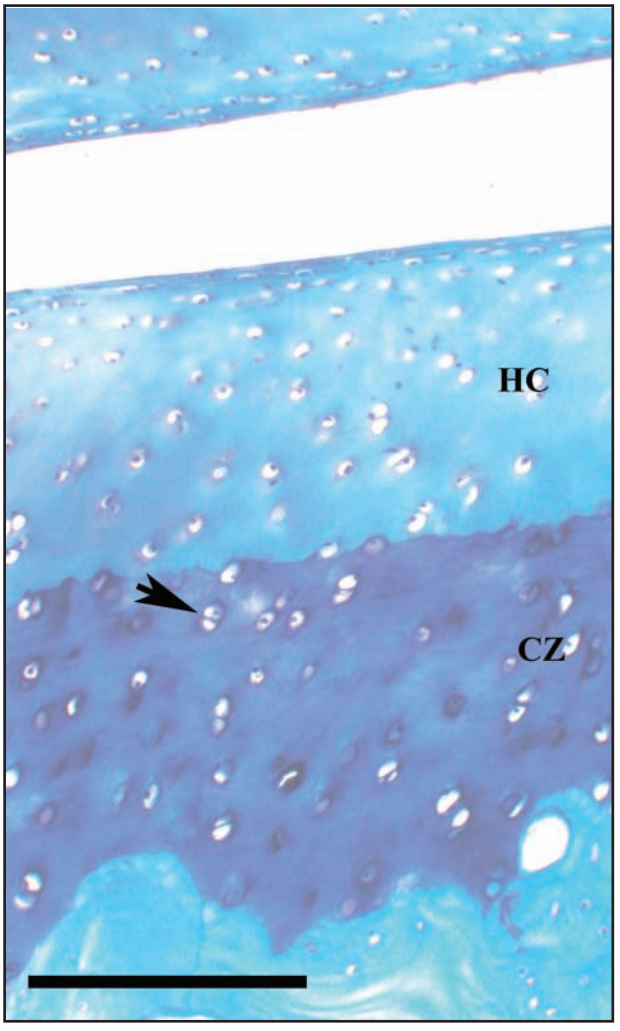

Figure 4—Photomicrograph of a tissue section obtained from the articular surface of the proximal aspect of the third metatarsal bone of a 3-year-old pony. The CZ is a thick distinct zone with a clearly defined tidemark and smaller chondrocytes. Notice that there are some doublet chondrocytes (arrow). The HC is relatively thinner. Toluidine blue stain; bar = 500 µm. *See* Figures 2 and 3 for remainder of key.

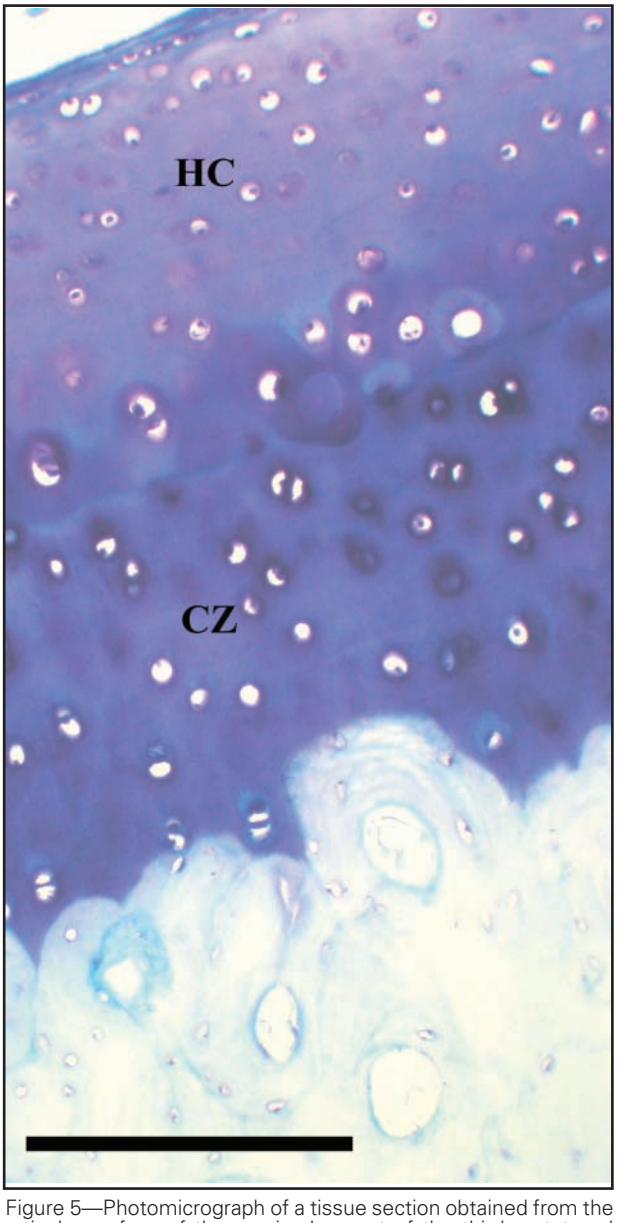

Figure 5—Photomicrograph of a tissue section obtained from the articular surface of the proximal aspect of the third metatarsal bone of an 8-year-old pony. The HC is thinner than in the younger ponies, and the CZ is almost as thick as the HC. Toluidine blue stain; bar = 500 µm. *See* Figures 2 and 3 for remainder of key.

Results

Description of hyaline cartilage and calcified cartilage—At 11 days of age, there was a thick layer of highly cellular hyaline cartilage with a smooth articular surface but without a distinct zone of calcified cartilage (**Figure 2**). The upper layers of the cartilage had a high density of small- to medium-size chondrocytes, with the most superficial layer (2 to 3 cells thick) arranged in parallel with the surface. However, the deeper layers, which were densely populated with chondrocytes, did not have a distinct anatomic alignment, although cells were evenly distributed within the matrix. The chondrocytes became larger toward the osteochondral junction, where there were large clusters of chondrocytes (typically had a corn-on-the-cob appearance) within a zone of active proliferation. Vascular spaces representing osteoclastic remodelling units extended into the zone of cartilage proliferation between forming bone trabeculae throughout the length of the osteochondral junction.

At 6 to 9 months of age, there was also a thick layer of hyaline cartilage and a smooth articular surface. A calcified zone was becoming apparent, but there was still active cartilage proliferation with clusters of large chondrocytes (**Figure 3**). The calcified zone was still invaded by osteoclastic remodelling units, although they were less prominent than those for the 11-day-old group, with fewer osteoclasts observed. Groups of smaller chondrocytes were aligned perpendicularly to the surface in the lower half of the hyaline cartilage. There was a lower cell density and a greater percentage of matrix, compared with results for the upper layers, which had a higher chondrocyte density and the most superficial layers of cells aligned parallel to the surface.

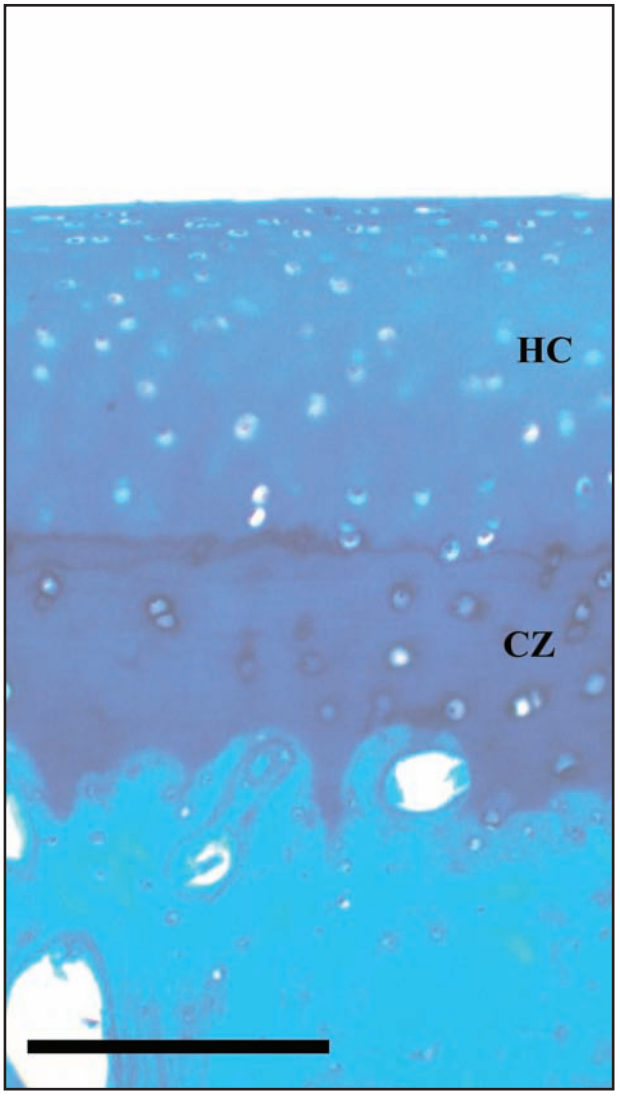

Figure 6—Photomicrograph of a tissue section obtained from the articular surface of the proximal aspect of the third metatarsal bone of a 12-year-old pony. There is reduced cellularity in the HC and CZ, compared with results for younger ponies. Toluidine blue stain; bar = 500 µm. *See* Figures 2 and 3 for remainder of key.

At 2 to 3 years of age, there was a distinct, thick calcified zone, whereas the hyaline cartilage was thinner than in the younger groups (**Figure 4**). There was no invasion of the calcified zone by osteoclastic remodelling units. The articular surface was not entirely smooth; instead, it had mild superficial undulations and indentations in places. Although chondrocytes were quite frequent within the calcified zone, they were much smaller than those in the 11-day-old or 6- to 9-month-old groups and were of similar size to those in the hyaline cartilage. However, chondrocytes in the calcified zone were frequently detected as doublets (a cluster of 2 cells), whereas chondrocytes in the hyaline cartilage were more frequently single cells with bigger nuclei. In the calcified zone, cells typically were aligned in columns at an angle to the osteochondral junction, near to and toward the periarticular edge. There was a thin layer of horizontally aligned cells in the most superficial part of the articular surface.

At 6 to 8 years of age, hyaline cartilage was thinner than that in the younger groups, whereas the calcified zone was thicker with relatively fewer chondrocytes and fewer chondrocyte doublets (**Figure 5**). Chondrocytes in the deeper part of the hyaline cartilage adjacent to the tidemark were medium in size, with chondrocytes becoming smaller toward the surface. The most superficial part contained a narrow layer (1 to 2 cells deep) of horizontally aligned cells. In places, the surface was slightly undulant, and cell density in these places typically was higher than the cell density elsewhere within the hyaline cartilage. However, in general, there was a fairly even distribution of cells within the matrix. Chondrocytes typically were aligned in columns at an angle to the osteochondral junction, near to and toward the periarticular edge.

At 11 to 17 years of age, the hyaline cartilage was thick at the dorsal extent and became thinner farther plantar. The calcified zone was still clearly defined and almost as thick as the hyaline cartilage (**Figure 6**). The density and arrangement of chondrocytes in the hyaline cartilage and calcified zone were similar to those in the 6- to 8-year-old group. In places, the articular surface undulated.

At 20 to 25 years of age, the hyaline cartilage varied in thickness throughout the joint, with undulation of the articular surface. The calcified zone was still clearly defined. The density and arrangement of chondrocytes were similar to those in the other mature groups of ponies.

Tidemark—A tidemark between the hyaline cartilage and calcified zone could not be defined at 11 days and was indistinct at 6 to 9 months of age. However, it was clearly defined in the older groups and was a clear boundary between the hyaline cartilage and the calcified zone.

Orientation of collagen fibers—Examination of the cartilage by use of polarized light revealed collagen fibers were orientated approximately parallel to the articular surface and were most distinct in the deeper layers of cartilage. At 6 to 9 months of age, collagen fibers appeared to be orientated at approximately 60° to the articular surface, with the angle from the dorsal to the plantar aspect of the joint. Toward the plantar aspect of the joint, fibers were sloping in a plantar to dorsal direction. In the most superficial part of the hyaline cartilage, there was a thin layer of fibers that were parallel to the articular surface. At 2 to 3 years of age, collagen fibers were almost perpendicular to the articular surface with some intersite variation in orientation. Fibers were arranged at an angle in opposing layers of hyaline cartilage. At 6 to 8 years of age, there was a similar appearance, whereas the 11- to 17-year-old ponies had more variation, with the fibers oriented at 45° to 60° to the surface in some places. Fibers sloped away from the dorsal aspect of the joint. Toward the plantar aspect of the joint, fibers were sloping in a plantar to dorsal direction. At 20 to 25 years of age, orientation of collagen fibers varied throughout the joint. Dorsally the fibers sloped from superficial to deep and from dorsal to plantar. Toward the plantar aspect, fibers were sloping from deep to superficial.

Osteochondral junction—At 11 days of age, the epiphysis appeared active with a cellular proliferative

calcified zone from which bone trabeculae were developing. At 6 to 9 months of age, there was still evidence of cartilage proliferation, but this had evidently ceased in the 2- to 3-year-old group. The region of the osteochondral junction had an irregular, indented profile in all groups.

Quantitative analysis of the association between age and osteochondral thickness—Total cartilage thickness decreased with increasing age, with a significant negative association detected at all sites ($r = -0.4$ to -0.54; $P = 0.002$ to 0.028), except for the lateral proximal aspect of the third metatarsal bone ($r = -0.31$; $P = 0.097$). For each site examined, there was a significant negative association ($r = -0.65$ to -0.84; $P < 0.001$) between age and thickness of the hyaline cartilage. There was a significant positive association ($r = 0.42$ to 0.65; $P < 0.001$ to 0.030) between age and thickness of the calcified cartilage. A significant positive association ($r = 0.69$ to 0.84; $P < 0.001$) between age and SCB thickness was detected at all sites.

Discussion

As hypothesized, morphologic characteristics of cartilage changed with age. Thickness of hyaline cartilage and total cartilage at a specific site decreased with increasing age, but thickness of calcified cartilage and SCB increased with increasing age, which supported our original hypotheses.

The ponies used in the study reported here were a unique group of animals that had been subjected solely to pasture exercise. This provided a sample of joints from a relatively controlled population in a natural environment, and load intensity was assumed to be similar throughout life. This provided an opportunity to study the effects of age with minimal superimposed effects of exercise.

The increase in SCB thickness, together with a relative increase in calcified cartilage and decrease in hyaline cartilage detected in our study, may have reflected accumulated load. High-intensity exercise has been associated with an advance in the calcified cartilage tidemark, with an increase in total cartilage thickness at sites without pathologic changes but a relative decrease in the thickness of hyaline cartilage and total cartilage at sites with osteoarthritic changes in horses[19] and dogs.[20] It has been postulated that microcracks in subchondral mineralized tissue lead to vascularization of calcified cartilage, reactivating the tidemark and bringing about thickened calcified tissues and a thinner hyaline cartilage.[21] Despite this, the greatest increase in SCB thickness was detected prior to skeletal maturity in the study reported here, so it was most influenced by the maturational process.

The SCB thickness increased with increasing age, with significant increases particularly evident among the immature groups. Substantial changes in biochemical composition of equine SCB take place during the first 5 months after birth and are an exercise-driven phenomenon.[2] It is also likely that in the study reported here, the increase in SCB thickness was an exercise-driven result. The SCB thickness continued to increase with increasing age after skeletal maturity. Changes in body shape and an increase in body weight could introduce changes in the loading pattern, which in turn could cause an increase in SCB plate thickness. The equine tarsus is subject to constant compressive loading,[22] and horses continue to load their joints throughout life, in contrast to humans who may become more sedentary in old age. Bone formation can be stimulated by a small number of strain cycles[23]; therefore, pasture exercise may be sufficient to stimulate changes to the SCB.

It is possible that the type of loading during pasture exercise is not adequate to stimulate thickening of the hyaline cartilage but that there are adequate intermittent high strains to stimulate the calcified tissues of joints. When material properties of bone remain similar with increases in age,[24] then an increase in thickness of the osteochondral calcified tissues with increasing age would lead to increased structural stiffness and an increased requirement for energy absorption by the hyaline cartilage, which would provide a potential mechanism for increasing risk of osteoarthritis with increasing age. It has been suggested that there is a low rate of collagen metabolism in articular cartilage of adult horses, and damage to the collagen network cannot therefore be adequately repaired.[25] Failure of the thin hyaline layer to completely absorb impact potentially leads to microdamage in the calcified cartilage and SCB.[10]

In the study reported here, cellularity of the hyaline cartilage and calcified cartilage appeared to decrease with increasing age. Cellularity of articular cartilage in humans, rabbits, and cattle decreases during growth.[26] Chondrocyte arrangement also changed with increasing age. In the adult and geriatric groups, there were some chondrocyte clusters in both the hyaline cartilage and calcified cartilage. In the knees and shoulders of humans, chondrocytes in the deep layers of cartilage may proliferate to form multicellular clusters before degenerating.[26] Chondrocyte aging and associated loss of function have been related to an increase in the incidence of osteoarthritis with increasing age.[4] Variation in chondrocyte morphology, with flattened cells in the superficial zone and rounded cells in the deep zones, has been related to depth-dependent variations in the mechanical environment.[7] Analysis of results reported here indicated the same variation in chondrocyte morphology in all age groups.

In this study, there was considerable intersite variation in the orientation of collagen fibers. It was suggested that in regions in which loading is high, collagen fibers in the SCB are organized to create a 3-dimensional network that can resist compressive forces, whereas collagen fibers in regions that do not typically bear a load are more disorganized.[27] This change in orientation of collagen fibers may be a result of adaptive change to loading to increase the strength of the bone. Results of the study reported here agree with those of another study[28] in which it was found that the arrangement of collagen fibers in the deep layers of cartilage changed from a mesh-like structure in young rabbits to a perpendicular arrangement in adolescent and adult rabbits.

The articular surface in the stifle joints of rabbits becomes uneven in older animals, with variation among sites and animals.[26] Similar changes were found in the

ponies in the study reported here, with a smooth articular surface in the unloaded neonatal and loaded immature groups, which subsequently became rough and undulated with indentations in the older groups. The osteochondral junction was irregular and interdigitated with the bone located underneath it in the neonatal and loaded immature groups; the osteochondral junction subsequently became more regular and extended far less into the bone in the loaded mature, adult, and aged groups. The junction between cartilage and bone is important because it has to compensate for a sudden change in mechanical properties. In an immature joint, the osteochondral junction is more susceptible to mechanical failure and collapse than in a mature joint because of subchondral shear stresses.[29]

The study reported here had some limitations. The group sizes were small, especially in the older age groups. Similar to humans, equids vary in the amount of exercise that they undertake. This may explain some of the variations within groups. The results are related only to the tarsometatarsal joint and are not necessarily transferable to other joints, especially those with different patterns of movement and loading.

In the study reported here, we detected changes in the thickness of hyaline cartilage, calcified cartilage, and SCB with increasing age as well as changes in adjacent related structures. Thickness of hyaline cartilage and total cartilage at a specific site decreased with increasing age, whereas thickness of calcified cartilage and SCB increased with increasing age, which may be related to relative responses of the tissues to strains induced by pasture exercise. However, the difference in response between the calcified and uncalcified layers could potentially have an influence on the resistance of the entire osteochondral unit to damage.

a. Biro UK, London, England.
b. Delta UK, Guiseley, England.
c. Cell Path PLC, Powys, Wales.
d. Ralwax, VWR, Poole, England.
e. Bayer VIP, Newbury, England.
f. Shandon Hypercut, Themo Shandon, Runcorn, England.
g. VWR, Poole, England.
h. DPX, Ray Lamd, Eastbourne, England.
i. Olympus DP12 microscope, Olympus UK Ltd, London, England.
j. Scion Image, Scion Corp, Frederick, Md.
k. Analyse-It for Microsoft Excel, version 1.73, Analyse-it Software Ltd, Leeds, England.

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