Effect of Loading on the Organization of the Collagen Fibril Network in Juvenile Equine Articular Cartilage

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Received 20 August 2008; accepted 28 January 2009
Published online 25 February 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20866

ABSTRACT: We investigated the effects of exercise-induced loading on the collagen network of equine articular cartilage. Collagen fibril architecture at a site (1) subjected to intermittent high-intensity loading was compared with that of an adjacent site (2) sustaining continuous low-level load. From horses exposed to forced exercise (CONDEX group) or not (PASTEX group), the spatial parallelism of fibrils and the orientation angle between fibrils and the surface at depths 9 μm apart through cartilage from surface to tidemark were determined using polarized light microscopy, and expressed as parallelism index (PI) and orientation index (OI). PI was significantly higher in site 2 than 1 in CONDEX and PASTEX groups. PI was significantly higher in forced exercised horses at site 2 but not site 1. OI was significantly greater (more perpendicular to the surface) in the superficial and deep cartilage of site 2 than 1 in both CONDEX and PASTEX groups. Superficial zone OI was higher in exercised horses at site 1 but not at site 2. Exercise increased collagen parallelism and affected orientation. The site differences in OI indicate that Benninghoff's classic predominantly perpendicular arcades appear not to be a consistent architectural feature, but adapt to local forces sustained. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1226–1234, 2009

Keywords: cartilage; collagen fibril; loading; parallelism; orientation

Different areas of articular cartilage are subjected to different degrees of loading. These conditions can be met adequately (without long-term damage) only by different cartilage areas possessing different mechanical properties, which are determined by the structural arrangement of its biochemical components.[1] Biochemical heterogeneity of equine articular cartilage has been shown extensively.[2,3] Specifically much work has been done comparing two sites on the proximal articular surface of the proximal phalangeal bone of the metacarpophalangeal (MCP) joint, which are known to be subjected to strongly differing loading conditions and to feature large differences in the incidence and manifestations of osteochondral disease.[4–6] Much less is known about possible topographical variation in the ultrastructure of equine articular cartilage. Benninghoff[7] used plane-polarized light microscopy to show the spatial organization of collagen network as a complex of arcade-like structures passing throughout three zones of hyaline cartilage. In the superficial zone, the predominant collagen fibril orientation is tangential to the articular surface, in the deep zone most fibrils are perpendicular to the calcified layer, and between is a transitional zone in which collagen orientation is more random.

In the newborn foal, there is evidence that topographical differences in the biochemical and (resulting) biomechanical properties of cartilage are not yet developed, or are substantially underdeveloped, and are acquired in the early postnatal period as a result of biomechanical loading.[8,9] There is also evidence from other species that the same may be true for the spatial organization of the collagen fibril network where the age of the subject, and frequency and mode of joint loading, appear to modulate the spatial architecture of the collagen network, especially in the superficial zone of articular cartilage.[10] Work in the horse is limited thus far to a preliminary study showing age-related changes in collagen fibril orientation in line with those described in other species.[11] The existence of a loading-regulated (re)modeling of the biochemical and/or ultrastructural characteristics of the collagen network in early life is of great importance given the incapacity of mature articular cartilage for considerable alterations and repair resulting from the extremely long turnover of collagen. Activity in early life thus may be a decisive factor for the biochemical and ultrastructural characteristics and, hence, biomechanical resistance to cartilage injury over the entire lifespan of the individual: Early exercise may be a crucial factor in the prevention of joint disease.[12,13]

The aim of this study was to investigate the effects of loading pattern and exercise intensity on the (ultra)-structure of the collagen fibril network in equine articular cartilage. It was hypothesized that there would be significant differences in collagen fibril architecture in two sites, subjected to either intermittent high-intensity loading or to more continuous low-level loading. A second hypothesis was that additional exercise superimposed on free pasture exercise during the first 18 months of life would affect collagen network architecture. To this end, a study using polarized light microscopy, allowing quantitative computation of the spatial collagen fibril parallelism and the orientation angle of the collagen fibril towards the articular surface,[14] was conducted on specimens from animals that formed part of a larger

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investigation into the influence of early exercise on musculoskeletal development and susceptibility to injury in horses.[15]

METHODS

Experimental Design

An extensive description of the set-up of the entire project is given by Rogers et al.[15] The project resulted from an international collaboration as a continuation on a previous study investigating the effect of exercise on the conditioning of the musculoskeletal system. Results from that study, suggesting a negative effect of sedentary lifestyle and sedentary lifestyle combined with short bouts of forced exercise on cartilage properties directed the design of the specific exercise regimen used in this study, in which an additional workload was superimposed on free pasture exercise.[16] Briefly, mixed sex (equally distributed) thoroughbred foals were assigned at birth to either a control group (PASTEX, $n = 15$), which exercised spontaneously at pasture only, or received additional exercise (CONDEX, $n = 18$) by being subjected to increasing exercise levels from approximately 10 days of age until 18 months of age (weaning was at 4 months). Exercise was given on a 515 m purpose-built track and consisted of 1,030 m each day, for 5 days a week. Initially, the foals exercised at a constant base speed of 5.4 m/s from an age of 21 ± 19 days until weaning at 138 ± 10 days, when the base velocity was increased to 7.5 m/s. At age 236 ± 31 days, base velocity was 9.6 m/s, with additionally a sprint over 129 m at 12.5 m/s after the first 500 m at base speed. The control group (PASTEX) were managed identically but exercised spontaneously at pasture only. The Cumulative Workload Index was 30% (significantly) higher in CONDEX than PASTEX foals.[15] At 18 months of age, 12 foals (6 from each group) were euthanized for postmortem analyses and used in the presented study. The animal experiment was approved by the institutional committee of animal welfare at Massey University New Zealand.

Sample Collection

The MCP joint of the left forelimb was isolated by transecting the proximal phalanx and the third metacarpal bone within 2 h of euthanasia and stored at -20°C until subsequent preparation of samples. After thawing, the joint was opened and inspected visually. Subsequently, osteochondral plugs (diameter 4 mm, 1–2 cm long) were taken perpendicular to the articular surface from two predefined locations on the proximal articular surface of the proximal phalangeal bone, using a custom-built hollow drill of which placement was directed by anatomical landmarks as described previously.[5] One plug was taken from the dorso-medial eminence (site 1, Fig. 1), which is exposed to dynamic, intermittent peak-type loading (likely to be encountered during gallop and jumping) but not loaded during standing or while the animal is moving slowly when walking or trotting.[4] Another plug was harvested from the mid-region of the medial joint surface (site 2, Fig. 1), which is constantly loaded during standing and during locomotion at all velocities but with peak loads substantially lower than at site 1.[4]

Preparation of Tissue Sections

After fixation with 4% formaldehyde with 0.7 M phosphate buffer (pH 7.0) at ambient temperature for 48 h, samples were decalcified in 10% EDTA supplemented with 4% formaldehyde and 0.1 M sodium phosphate buffer (pH 7.4) for 12 days at 4°C. The specimens were dehydrated in ascending series of alcoholic solutions and embedded in Paraplast Plus® wax (Sherwood Medical, St. Louis, MO). Unstained 7-μm sections were cut perpendicular to the cartilage surface (LKB 2218 Historange microtome; LKB-Produkter Ab, Bromma, Sweden), from two orthogonal section planes. Sections were dried overnight at 37°C, treated with xylene at 37°C for 24 h, and taken through a descending alcohol series. To remove any masking glycosaminoglycans, the dewaxed 7-μm sections were digested with bovine testicular hyaluronidase solution (Sigma Chemical Corporation, St. Louis, MO) and left unstained prior to DePex embedding (BDH, Poole, UK) and polarized microscopy.[14,17]

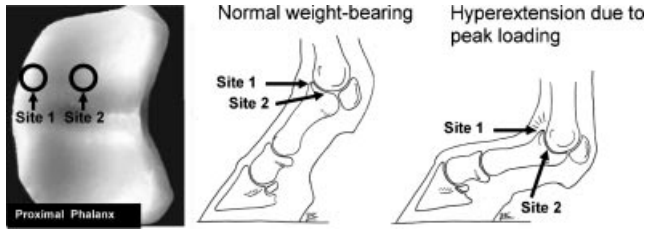

Figure 1. Sampling sites of cylindrical osteochondral plugs for polarized light microscopy from the proximal articular surface of the proximal phalangeal bone of the left metacarpophalangeal joint. Located at the medial dorsal margin of the joint surface, site 1 is not loaded during standing or in a slowly moving animal but is subjected to high intermittent peak loading at high speeds and jumping. Site 2, in the medio-central area, is continually loaded when the limb is weight-bearing, but experiences lower peak forces than site 1.

Quantitative Polarized Light Microscopy

A methodological description of the used quantitative polarized light microscopy technique has recently been published by Rieppo et al. with a detailed technical appendix.[14] In short, the measurement system consists of a Leitz Ortholux BK-2 polarized light microscope (Leitz, Wetzlar, Germany) modified for computation of Stokes parameters, a $2.5\times$ strain-free objective, a Peltier-cooled high-performance CCD camera (Photometrics SenSys, Roper Scientific, Tucson, AZ), and computer-controlled stepwise rotating crossed polarizers. Accurate quantification of the characteristic behavior of linearly polarized light can be made only under monochromatic circumstances, therefore, wavelength-matched optical components and precision grade polarizers were used. Two separate monochromators [591.4 ± 10 nm (Schott, Mainz, Germany) and 594 ± 3 nm XLK10 (Omega Optical Inc., Brattleboro, VT)] were used to adjust the final measuring wavelength ($\lambda = 594 \pm 3$ nm) of a halogen lamp powered by a 135W AC/DC switch mode Mascot 9522 power source (Mascot Electronics AS, Frederikstad, Norway). Highly linearly polarized light was used as an optical probe for the characterization of collagen orientation and parallelism. The two polarizers and a $\lambda/4$ phase shifter of the system were specifically manufactured for the operation at 594 nm wavelength (10LP-VIS, 10RP34; Newport Corporation, Irvine, CA). Starting from the alignment position (= 0°), 15° stepwise rotations were made of the polarizer pair. Thus, images were recorded at 0°, 15°, 30°, 45°, 60°, 75°, and 90° polarizer pair positions, and an additional 90° image was taken after placing the $\lambda/4$ phase shift plate into the light path. Images were then rescaled to a lower resolution, and 5×5 median filtering and exclusion was done to avoid the effects of different cell densities and cell lacunae on the final calculations. The collagen fibril parallelism and orientation angle distributions

were computed on a pixel basis starting from the articular surface and ending at the osteochondral junction using 9-μm spatial resolution (pixel size, 9.01×9.01 μm) between the points measured, i.e., articular surface to tidemark. Pixel-by-pixel, background-corrected orientation independent birefringence images of the collagen network of the tissue sections were created that were used for calculation of parallelism and orientation of collagen fibrils. Intensity values of each pixel recorded after stepwise rotation of polarizers can be fitted to the $\sin^2$–function. Since the function reaches its maximum and minimum every 45° interval, it is possible to calculate its real minimum and maximum value. The parallelism of the fibrils was calculated from the maximum and minimum signals determined using the least square fitting procedure for each image pixel. When there is only a single fibril or multiple fibrils running in the same direction, the observed signal reaches zero at a specific angle when it is measured with linear polarized microscopy. This is an indication that the parallelism of the collagen fibrils is high. When all possible fibril orientations are equally represented, the signal intensity remains constant (low parallelism). The parallelism index (PI; 0%–100%) characterizes the degree of collagen fibril parallelism. A high PI indicates that most collagen fibrils in the pixel in question run in the same direction; a low PI represents tissue with more randomly arranged collagen fibrils. An extreme PI value of 0% denotes the lack of fibril parallelism.

The orientation of collagen fibrils was calculated from the Stokes parameters by determination of the angle of the polarization ellipse. The orientation index (OI; 0°–90°) characterizes the angle between the joint surface and the measured average lengthwise orientation of collagen fibrils in the cartilage section. For analysis, tissue sections were positioned in the microscope according to the cartilage surface which was defined as 0° direction. The direction at right angles to this was defined as 90° direction. The OI expresses the actual orientation of the collagen fibrils towards the articular surface, i.e., at the extremes: OI of 0° means collagen fibrils are parallel to the articular surface, OI of 90° indicates collagen fibrils are perpendicular to the articular surface. The spatial coordinate system for collagen orientation is shown in Figure 2A.

From the tissue specimens, sections were analyzed in two planes cut at right angles to each other. Three randomly selected unstained serial sections were analyzed and averaged for each of the two cutting planes to reduce any possible bias arising from the slightly variable section thickness. For comparison of data, averages of the measurements per sample from each site were used. For image capture, processing, analysis, and macro-routines of data calculation, IPLab Spectrum (BD Biosciences Bioimaging, Rockville, MD) and Microsoft Excel (Microsoft Corporation, Redmond, WA) software were used.

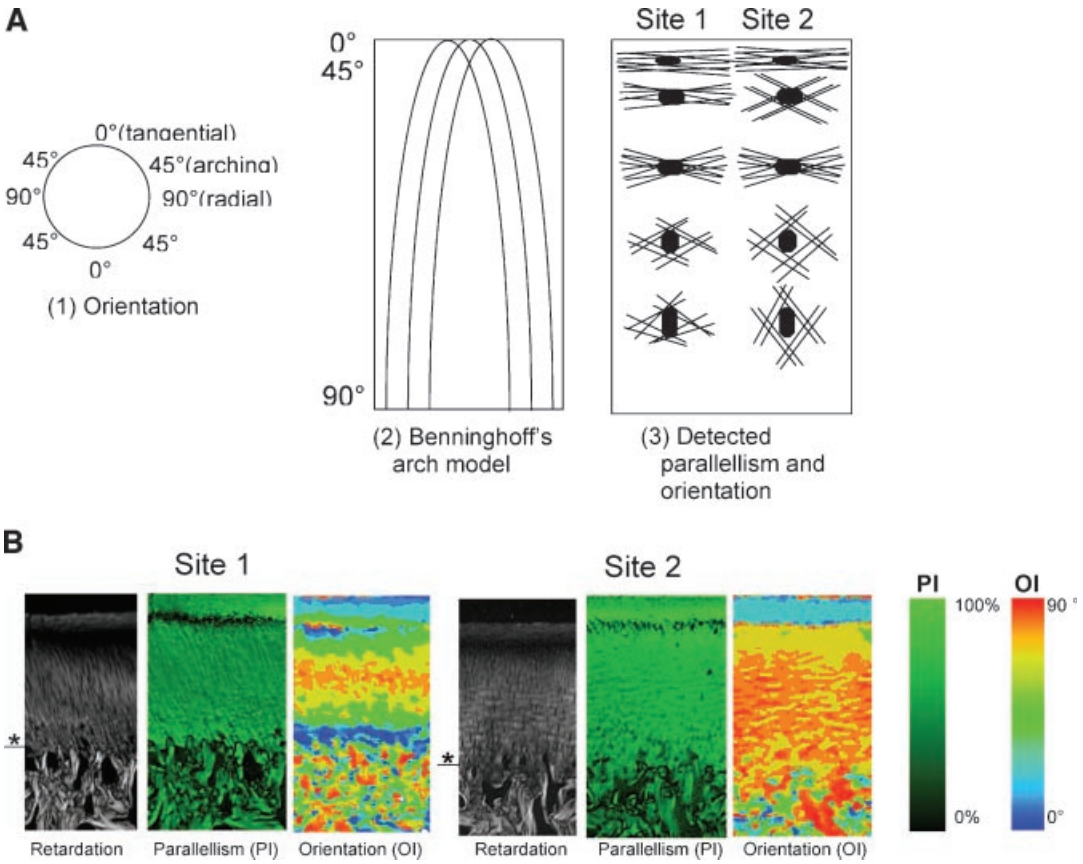

Figure 2. Visualization of polarized microscopy measurements of structural characteristics of articular cartilage collagen network. (A) Spatial orientation of collagen (in degrees) (1). Hypothetical arch model for the collagen fibril bundles emerging from the cartilage surface to the osteochondral junction according to Benninghoff (2). Schematic model of parallelism and orientation of the collagen in the different zones of articular cartilage on site 1 and site 2. The orientation angles and degree of parallelism have been extrapolated from the results of PASTEX and CONDEX groups (3). (B) Parallelism (PI) and orientation index (OI) images of site 1 and site 2 of a PASTEX group horse. Osteochondral junction and anatomical landmarks are indicated in retardance images obtained with least square method. The cartilage surface and the height of the osteochondral junction are comparable.

Data Presentation and Statistical Analysis

Data are presented as mean ± standard deviation (SD) and visualized in a spatial arrangement of 9 μm steps (absolute units) from articular surface to the tidemark at the osteochondral junction. Alternatively, a percentage of total thickness could have been used for presentation. However, thickness of the transitional and deep zones that make up most of the cartilage depth may differ according to anatomical location,[18] in which case results expressed by absolute depth will provide more factual information. For comparison of the results from sites 1 and 2, nonparametric Wilcoxon matched-pairs signed-ranks test was used, and Mann–Whitney's *U*-test with Bonferroni adjustment for comparison of CONDEX and PASTEX groups, using the SPSS 12.0.2 software package (SPSS, Chicago, IL), with statistical significance set at 0.05.

RESULTS

The macroscopic appearance of articular cartilage from the two anatomical sites of both CONDEX and PASTEX animals was normal. Figure 2A,B visualizes polarized microscopy measurements of structural characteristics of articular cartilage collagen network both schematically and through microphotographs from site 1 and 2. In Figures 3A,D and 4A,D, PI and OI, respectively, are visualized in a spatial arrangement of 9 μm steps (absolute units) from articular surface to the tidemark at the osteochondral junction.

Collagen Fibril Parallelism

The PI varied with cartilage depth in all specimens analyzed. In samples from PASTEX foals, collagen

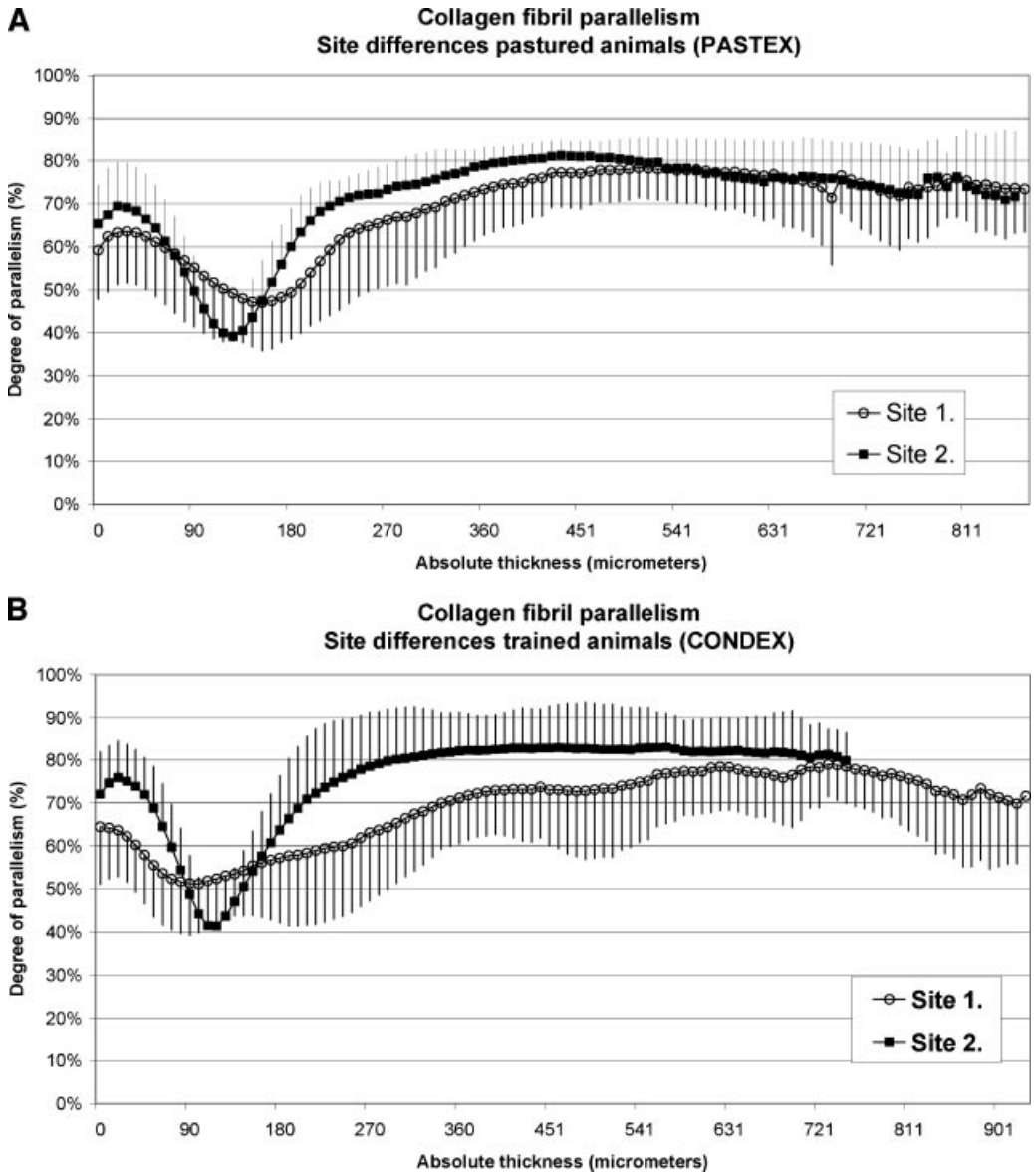

Figure 3. The average degree of collagen parallelism (PI; mean ± SD) over the entire depth of the cartilage, measured with quantitative polarized light microscopy at points 9 μm apart from each other from the cartilage surface to the osteochondral junction. A PI of 0% indicates random course of fibrils, and 100% indicates complete parallelism. (A) Pastured animals (PASTEX); (B) exercised animals (CONDEX); (C) site 1 PASTEX and CONDEX groups; (D) site 2 PASTEX and CONDEX groups.

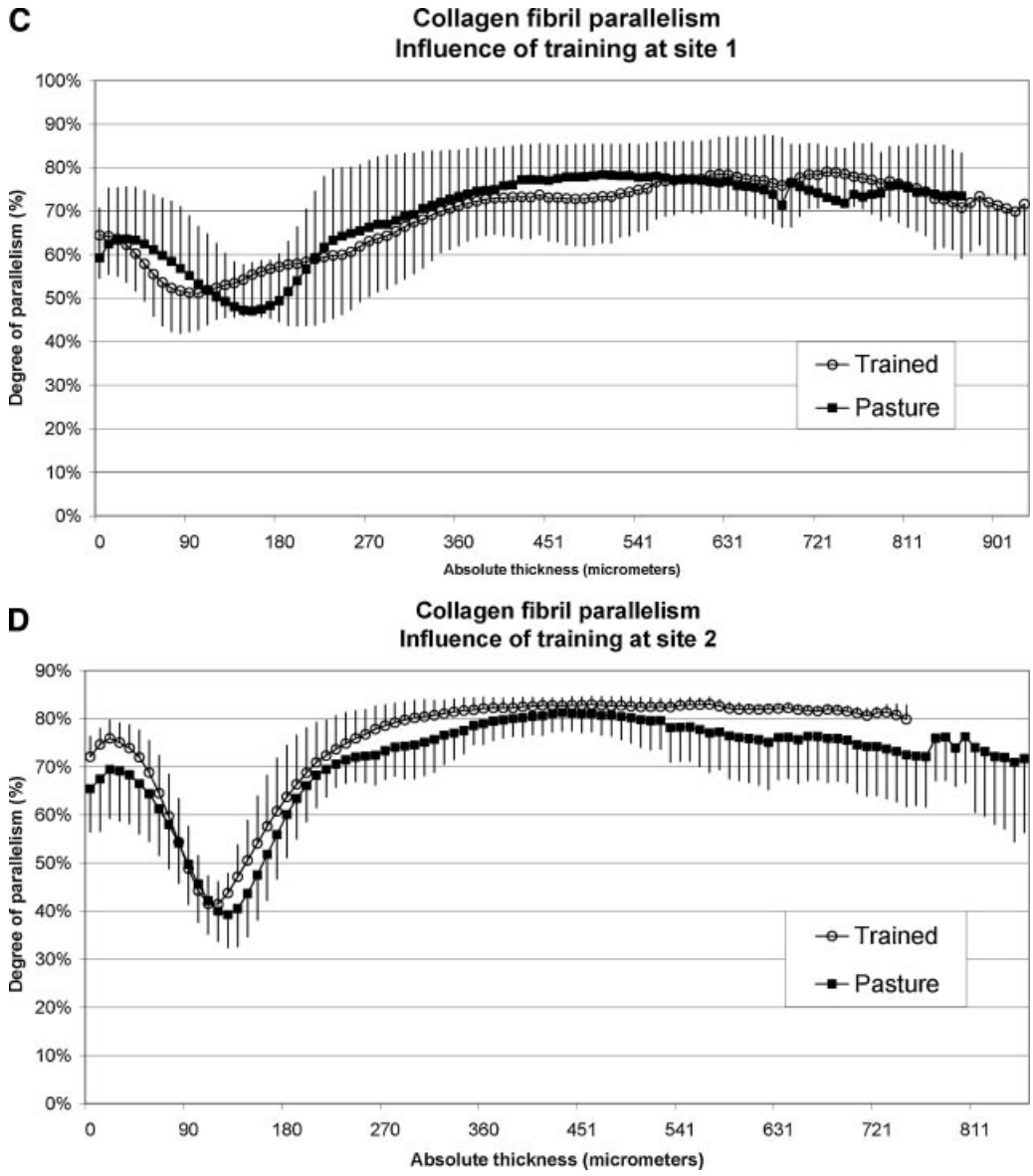

Figure 3. (*Continued*)

fibrils in the superficial zone of site 1 had a PI of 60%–65%, which slowly decreased to 48% at a depth of approximately 175 μm (Fig. 3A). Deeper towards the osteochondral junction, the PI increased to almost 80% at a depth of approximately 500 μm, reaching a plateau value that continued to the osteochondral junction. A more or less similar general pattern (high-low-high) was found at site 2. However, at various depths, absolute PI values differed significantly between sites 1 and 2. At site 2, the superficial cartilage zone had a significantly higher PI, but a significantly lower PI at a depth of 100 μm compared to site 1. Also, there was a more abrupt increase towards the plateau, which was already reached at a depth of 300 μm. In the CONDEX group, a grossly similar high-low-high pattern was seen (Fig. 3B). Again, significant differences were demonstrated between sites 1 and 2. There were no differences in the overall patterns of collagen fibril parallelism at site 1 between tissues from PASTEX and CONDEX animals (Fig. 3C). At site 2, the PI of the CONDEX animals was consistently higher throughout the superficial cartilage layers until approximately 80 μm depth and after a depth of 120 μm (Fig. 3D).

Collagen Fibril Orientation

The OI also varied with cartilage depth in all specimens analyzed, but sites 1 and 2 demonstrated very different patterns. In the PASTEX group, OI at site 1 slowly increased with increasing depth from approximately 25° to 40° at a depth of 100 μm (Fig. 4A). From that depth, OI decreased slowly to approximately 30° at 500 μm, followed by a slow increase to approximately 45° in the deepest cartilage layers. OI at site 2 started at the same value, but increased much more rapidly in the superficial cartilage layers to 50° in less than 100 μm depth. From there, OI declined to approximately 30° at

200 μm depth, and subsequently increased towards 50° at approximately 600 μm, at which value it remained until the osteochondral junction. In the CONDEX group, there was less variation at site 1: OI fluctuated between approximately 30° to 40° until approximately 600 μm depth (Fig. 4B), after which it slowly increased to approximately 50° at the osteochondral junction. OI followed a significantly different pattern at site 2 where there was a relatively sharp increase of OI in the superficial cartilage zone from approximately 30° to 60° over less than 100 μm depth, followed by a decline to approximately 25°–30° at 180 μm and a slow increase towards 50° at approximately 600 μm, after which OI remained constant until the osteochondral junction is reached. There were significant differences in OI when comparing PASTEX and CONDEX animals at the two sites (Fig. 4C). In the most superficial 90 μm, the OI was higher in the CONDEX than the PASTEX group, while at greater depths OI was higher in the PASTEX tissues. At site 2, there were no significant differences in OI between PASTEX and CONDEX animals (Fig. 4D).

DISCUSSION

This study used polarized light microscopy that has traditionally been used to visualize the structural properties of the collagen network.[19,20] It provides a wide view over the tissue section and allows for a straightforward correlation of the results with other histological methods. Traditional plane-polarized techniques cannot overcome the problem of unequal detection sensitivity of differently oriented structures, but recently, with the advent of computerized

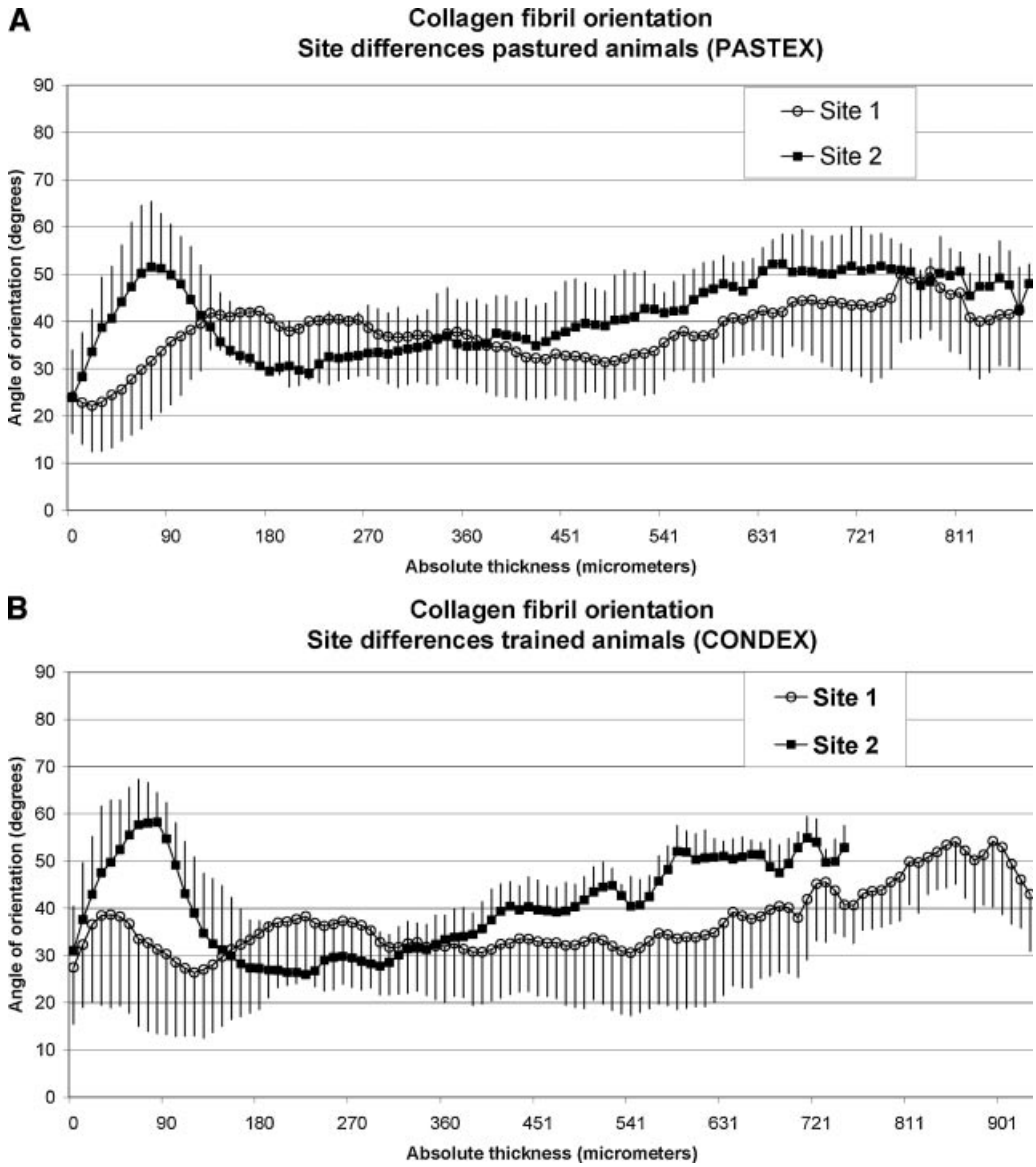

Figure 4. The average degree of collagen orientation (mean ± SD) over the entire depth of the cartilage, measured with quantitative polarized light microscopy at points 9 μm apart from each other from the cartilage surface to the osteochondral junction. OI (see text) of 0° indicates fibril orientation parallel with the cartilage surface and 90° is perpendicular orientation to the cartilage surface. (A) Pastured animals (PASTEX); (B) exercised animals (CONDEX); (C) site 1 PASTEX and CONDEX groups; (D) site 2 PASTEX and CONDEX groups.

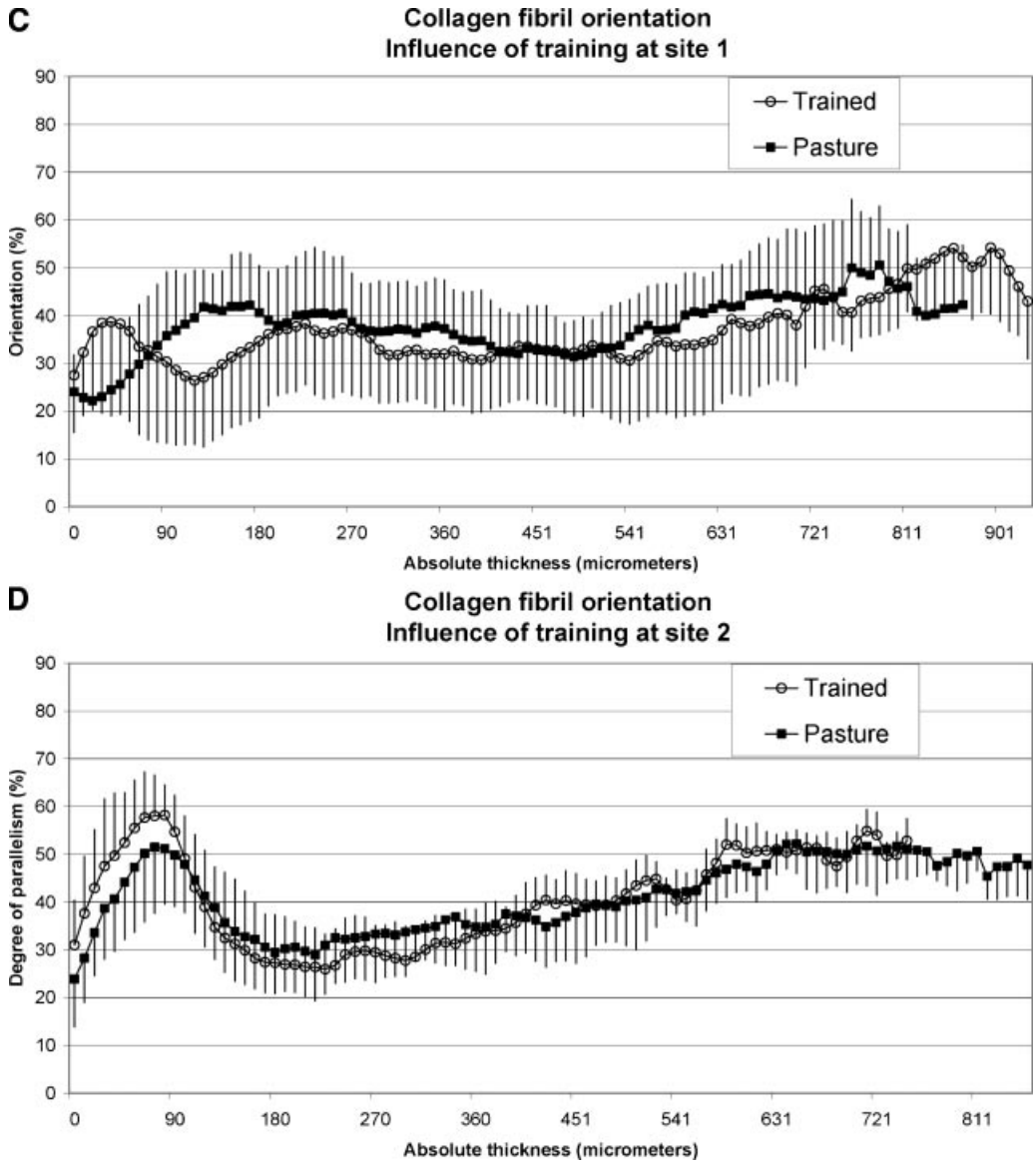

Figure 4. (*Continued*)

instrumentation of microscope polarizers, and combining data from separate images with Stokes' calculus, it has proved possible to acquire structural collagen data independently of the fibril orientation.[14] This new, profoundly modified quantification of the polarized microscopic phenomena, used in this study, allows greater precision than traditional methods, avoids the effect of section thickness, and enables the quantitative pixel-by-pixel analysis of PI and OI, which was not possible with the traditional techniques.

Joint surfaces are not loaded uniformly during locomotion, nor are the loads experienced throughout the depth of the cartilage identical.[4,21] Different sites in a particular cartilage surface subjected to distinct loading patterns have different biochemical composition,[3–5] and the present study shows that there are also important differences in collagen structure between these sites: Irrespective of exercise group, there were significant differences in both parallelism and orientation of collagen fibrils between sites 1 and 2. PI was higher centrally (site 2) than peripherally (site 1), corresponding with marginal areas having a much larger transitional zone with more random fibril orientation than did central areas.[18] Also, OI was different between sites 1 and 2. At site 2, an interesting phenomenon was observed: As expected, the OI was low superficially and increased in the transitional zone until a depth of approximately 100 μm. From that point, there was a decrease in OI until approximately 180 μm, from where the OI increased to approximately 50°. This is not in agreement with the classic Benninghoff model recently confirmed in pigs,[22] in which OI remained stable at an average of 76° from a depth of about 200 μm, corresponding to the end of the transitional zone. This discrepancy is explained by either the collagen network of the 18-month-old thoroughbred not yet having reached its

final configuration, or the OI in the deep zone is not 90° to the subchondral bone, but less. The first option seems improbable given the high degree of parallelism seen at this site.[22] A more likely explanation is an adaptation of the direction of collagen fibers to high shear forces in the equine MCP joint, which is a heavily loaded joint with an exceptionally high range of motion where an oblique insertion of the collagen fibers in the calcified cartilage layer may yield better resistance to repeated shear forces than would a perpendicular configuration. It may be hypothesized, therefore, that the Benninghoff arcade model may be more flexible than commonly thought and that local adaptations may include a predominantly nonperpendicular direction of the collagen fibrils in the deep zone.

Literature on the effect of exercise on the ultrastructural characteristics of the cartilage collagen network is sparse. Lower birefringence in the superficial zone of young beagles after long distance running indicated disorganization or reorganization of collagen, but the exercise level was very high (40 km/day).[23] Exercise improved collagen structure in young guinea pigs, but resulted in deterioration in older ones,[10] in agreement with formation of a Benninghoff-type architecture within a species-specific timeframe.[11,22,24,25] We also show a higher degree of organization (PI was significantly higher) at site 2 in the CONDEX group. Although a gradual increase in fibrillar organization is part of age-related maturation,[22] the exercise in the juvenile thoroughbred horses advanced the normal process of increasing ultrastructural organization, corresponding with advancement of age-related changes in biochemical composition in the same group of horses.[26] The induced difference was rather small. This could be expected, given the relatively modest increase in workload of 30%.[15] The level of exercise in the control group can be seen as a natural baseline level, as it has been shown that foals enjoying free pasture exercise are subjected to a workload that is similar to the workload of feral horses.[27] However, even this slight effect of exercise on the superficial collagen fibril network can be anticipated to increase the dynamic modulus of the cartilage when investigated with the indentation test.[28] Increase of the dynamic modulus would witness of stiffer cartilage, capable of sustaining higher loads.[28,29] The biomechanical effect of the changes in the collagen network may be enhanced by concomitant changes in glycosaminoglycans (GAG) and total collagen content, as demonstrated earlier in the same group of animals at site 2. The GAG/collagen ratio was significantly higher in the CONDEX animals at that site, which, together with the increased cross-linking in the CONDEX group, might be indicative of a cartilage extracellular matrix that features both increased tensile and compressive strength.[26]

There was no effect of exercise on PI at site 1, possibly due to site 1 being only intermittently loaded and the total effect of the additional workload thus being less. Exercise-induced changes in biochemical composition were also more evident at site 2 than at site 1.[26] Another explanation is that, due to the marginal location and more occasional loading of site 1, this site will retain a less organized collagen structure anyhow.

Collagen fibril orientation was not influenced by exercise at site 2, but there was a small yet significant effect at site 1. At that site, a significant increase in OI was noted in the exercised group in the superficial zone. It is tempting to speculate that this higher OI level in the superficial zone might increase resistance to shear loading at the margin of the articular surface, which could be seen as a beneficial effect in the CONDEX group.

It can be concluded that early additional exercise has site-specific significant effects on collagen structural properties of cartilage in the foal. The future susceptibility to injury could potentially be influenced by means of well-tailored exercise regimens, which will be of most interest in those species (human, horse) in which long-term orthopedic health is a serious concern.[13,30] Having shown that even mild exercise influenced structural and biochemical characteristics positively, it is unclear if this type of manipulation is beneficial in the long-term. Exposure to the challenges that will be faced during an athletic or working career at a young age, when collagen configuration is still being shaped, may positively influence provision of the type of collagen that is best adapted and, hence, least susceptible to injury. In the last decade, structured school-based exercise programs have been introduced in children, with no apparent problems and showing that early puberty may be a particularly opportune time during growth for simple exercise interventions to have a positive effect on bone health.[31] We show that in animals exposed to exercise, at a similar stage of life as young children, there was a positive effect on collagen architecture in cartilage. On the other hand, it may be argued that speeding up the physiological maturation process will advance the age after which remodeling of cartilage becomes virtually impossible and with it the capacity for repair, which might be a disadvantage. Only long-term follow-up of sufficient numbers of mammals that have undergone (very) early exercise programs can answer this question.

ACKNOWLEDGMENTS

We thank Mrs. Eija Rahunen and Mr. Kari Kotikumpu for their skillful technical help. Financial support from Academy of Finland, Ministry of Education Finland, National Graduate School of Musculoskeletal Diseases Finland, Science Foundation Ireland, and the New Zealand Racing Board is gratefully acknowledged.

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