Effect of exercise on age-related changes in collagen fibril diameter distributions in the common digital extensor tendons of young horses

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Objective—To determine whether specific treadmill exercise regimens would accelerate age-related changes in collagen fibril diameter distributions in the common digital extensor tendon (CDET) of the forelimbs of young Thoroughbreds.

Animals—24 female Thoroughbreds.

Procedure—Horses were trained for 18 weeks (6 horses; short term) or 18 months (5 horses; long term) on a high-speed treadmill; 2 age-matched control groups (6 horses/group) performed walking exercise only. Horses were (mean ± SD) 24 ± 1 months and 39 ± 1 months old at termination of the short-term and long-term regimens, respectively. Midmetacarpal CDET specimens were obtained and processed for transmission electron microscopy. Diameter and area of at least 1,000 collagen fibrils/specimen were measured by use of computerized image analysis. Mass-average diameter (MAD) of collagen fibrils and collagen fibril index were calculated for each horse.

Results—Collagen fibril MAD for the older horses was significantly less than that for the younger horses. Exercise did not significantly affect fibril diameter or distributions in either age group, and collagen fibril index did not differ significantly between groups.

Conclusions and Clinical Relevance—Age-related reduction in collagen fibril MAD agreed with findings for other tendons and species. Training did not accelerate age-related change in the CDET, in contrast to a reported decrease in collagen fibril MAD in the superficial digital flexor tendon of horses trained long term. Our results support the concept that the functionally distinct nature of the CDET and superficial digital flexor tendon in horses results in fundamentally different responses to high-speed exercise regimens. (Am J Vet Res 2005;66:564–568)
a greater potential for interfibrillar cross-links that are proposed to inhibit creep between fibrils and confer elasticity. Therefore, the proportions of large- and small-diameter collagen fibrils should be associated with strength and elasticity of the fascicles and tendons as a whole. The fibril population may be described by the collagen fibril index (CFI), which is the proportion of a given area of tendon matrix that they occupy. The diameter distribution is most usefully described by the mass-average diameter (MAD) of collagen fibrils, which is effectively the mean of the fibril diameter-area distribution as derived from the diameter-frequency distribution of a given fibril population. The MAD takes into account the fact that a small number of large fibrils make a substantial contribution to the tensile strength, and this variable has been positively correlated with tensile strength during in vitro tests. In numerous tendons (including the calcaneus tendon in humans; calcaneus, flexor, and tail tendons in rats; and digital extensor and flexor tendons in sheep), the MAD reportedly increases during maturation and thereafter decreases with age as a result of loss of the largest fibrils. Age-related MAD data for the SDFT and CDET of horses are limited but indicate a similar pattern with skeletal maturation at 2 to 3 years of age.

Few studies have documented adaptation of tendon tissue to exercise. In a study, apparent hypertrophy was documented in the CDET of exercised horses; the cross-sectional area of the CDET of Thoroughbred fillies that were 24 months old at the end of an 18-week exercise regimen on a high-speed treadmill approached that of a group of 3-month-old fillies that had been involved in an 18-month exercise regimen. Analysis of these results indicates that training on the treadmill accelerated a typically age-related process in the younger horses. A CDET with a larger cross-sectional area in young horses would be expected to be stronger. However, in the older horses of the 18-month study, there was a detrimental effect of exercise on the energy-storing SDFT characterized as a reduction in collagen fibril MAD in the center of a cross section of the SDFT in the midmetacarpal region caused by apparent breakdown of existing fibrils. This was interpreted as acceleration of an age-related change in the distribution of fibril diameter and also as microtrauma because a lower MAD implies reduced tensile strength of the matrix in that injury-prone tendon site.

We hypothesized that there would be an age-related reduction in the collagen fibril MAD of the positional CDET that would be accelerated by exercise in a similar manner to that reported for the SDFT. The objective of the study reported here was to determine collagen fibril MAD values for the CDET of young Thoroughbreds subjected to exercise on a treadmill in accordance with specific 18-week and 18-month training regimens and age-matched control horses.

Materials and Methods

Horses and training regimens—Twelve young female Thoroughbreds were used in 2 experiments (a long-term and short-term exercise regimen). Mean ± SD age of horses for the long-term exercise regimen was 21.3 ± 1.1 months, whereas mean age of the 6 horses for the short-term exercise regimen was 19.4 ± 0.6 months. There were 6 age- and sex-matched control horses for the long-term (mean, 20.7 ± 1.1 months) and short-term (mean, 19.2 ± 1.2 months) exercise regimens. Horses were allocated randomly to exercise or control groups.

Horses of the control and exercise groups for both exercise regimens were housed loose in box stalls (3.0 × 3.7 m). Horses were walked for 40 min/d, 6 d/wk on a mechanical walker. Control horses were walked to avoid effects of immobilization that may have developed had those horses been confined to box stalls. Only females were used in the study to ensure standardization of the groups; we are not aware of any evidence to suggest that sex would affect the incidence of tendon injury in horses. The protocols for this study were approved by the funding agency and the United Kingdom Government regulatory authority, the British Home Office, according to the Animals (Scientific Procedures) Act of 1986.

Exercise regimens—The long-term exercise regimen was conducted first, followed by the short-term exercise regimen. On the basis of advice from a local veterinary supervisor, some alterations were made in the short-term exercise regimen.

Horses were exercised on a high-speed treadmill at a slope of 3% to 4%. One of the horses in the long-term exercise regimen was withdrawn from the study because of illness during the training period. The training procedure for each exercise group in each of the exercise regimens was summarized (Appendix).

Collection and processing of tendon specimens—After completion of the exercise regimens, a segment (1 cm long) was harvested from the midpoint of the metacarpal region of the CDET of the left forelimb of each horse. Tendon samples were placed in labeled vials containing 2% glutaraldehyde in sodium cacodylate buffer and stored at 4°C for 24 hours. Longitudinal specimens (1 × 1 × 2 mm) were then excised from the midpoint of the tendon cross section by use of a scalpel blade; these specimens were placed in fixative for an additional 24 hours. Specimens were then processed for transmission electron microscopy and embedded in epoxy resin. Transverse sections of the embedded specimens were cut onto supported, 200-mesh copper grids. Grids were stained in 2% aqueous uranyl acetate followed by Reynold's lead citrate.

Electron microscopy and collagen fibril morphometry—Several sections of each tendon were examined, and a representative section was chosen for analysis. Micrographs were obtained of representative regions from 1 section/tendon. Sections were examined at 80 kV by use of a transmission electron microscope. Areas containing elastic fibers, cellular components, stain residue, and other artifacts were avoided. Negatives were developed, fixed, and then printed onto multigrade paper at a final magnification of 30,800X. Images were scanned by use of imaging software, and the resulting digital images were analyzed by use of a computerized image-analysis program. A fixed region of interest (ROI) of 20.6 μm² was imposed on each image, with exclusion of the borders and identification label. Measurements of diameter and area of fibrils within the ROI were automated. Automated computerized image-analysis processes will join some fibrils that are closely adjacent. Therefore, after the automated process, fibril margins that had been incorrectly joined were divided manually by drawing a line between them. Measurements within
the ROI were then repeated. Sufficient images to yield diameter measurements of at least 1,000 fibrils were analyzed. Typically, this required 3 to 5 images/specimen from fields that did not overlap.19

**Statistical analysis**—The CFI was calculated by dividing the area covered by fibrils in each ROI by the total area, and a mean value was then calculated for each tendon specimen. The MAD was calculated by use of the following equation:

$$\text{MAD} = \frac{\sum (n_i \times d_i^3)}{\sum (n_i \times d_i^2)},$$

where $n_i$ is the number of fibrils with a specific diameter and $d_i$ is that specific diameter. Linear regression was used to analyze all data; the dependent variable was defined as CFI or MAD, with independent variables of group (exercise or control group) and exercise regimen (long term or short term). The variable for exercise regimen corresponded to age (horses were 24 ± 1 months and 39 ± 1 months old at the completion of the short-term and long-term exercise regimens, respectively).

Stepwise regression (0.05 < $P$ < 0.1) was used to confirm significant differences. Significance was defined at values of $P$ < 0.05. Statistical analyses were performed by use of a statistical software package.4 All results were expressed as mean ± SD.

**Results**

**CFI**—The CFI ranged from 72.4% to 87.7% for the 4 groups. We did not detect significant differences among any of the groups.

**MAD**—We did not detect significant differences in MAD between exercise and control horses for the long-term ($P = 0.518$) or short-term ($P = 0.651$) exercise regimens (Table 1). The MAD for the younger horses in the short-term exercise regimen was significantly ($P = 0.047$) greater than that for the older horses in the long-term exercise regimen.

Because we did not detect significant differences between exercise and control groups for each exercise regimen, we combined the exercise and control groups, respectively, for the entire study for analysis. There was a greater number of fibrils $< 100$ nm in diameter for the older horses (30th percentile; 101.5 nm in diameter for older horses and 112.9 nm in diameter for younger horses) and a greater number of fibrils $> 300$ nm in diameter for the younger horses (80th percentile; 309 nm in diameter for younger horses and 280.1 nm in diameter for older horses).

**Discussion**

In the study reported here, we did not detect alterations in collagen fibril MAD in response to short-term or long-term exercise regimens. There are limited and conflicting data on the effect of exercise on distributions of collagen fibril diameter in tendons. Increases in collagen fibril diameter were measured in calcaneus tendons of rats subjected to a 10-week treadmill training regimen.20 Hind limb tendons of treadmill-exercised mice had an increase in mean fibril diameter after 1 week of exercise but a reduction from 3 to 7 weeks with evidence of longitudinal splitting of fibrils.21

However, information from other species may not be directly comparable to that for horses, given the functional and anatomic specialization of the distal portion of the limb in horses and the potential variations in loading patterns among various tendons.22 A significant reduction in MAD was reported in the SDFT core of horses trained by use of the same long-term exercise regimen used in the study reported; this is an injury-prone site. Such a reduction in MAD would theoretically result in a decrease in tensile strength and was suggested to be evidence of microtrauma.13 Similar reductions in collagen fibril diameter, which were interpreted as microdamage, were documented in cranial cruciate ligaments of exercised rats23 and caudal cruciate ligaments of dogs24 that were overloaded by surgical transection of the cranial cruciate ligaments. Exercise-induced reductions in collagen fibril diameter were also evident in cranial cruciate ligaments of bipedal rats; however, this was interpreted as evidence of increased synthetic activity rather than degeneration of existing fibrils on the basis of associated changes in morphologic characteristics of tenocytes including increased amounts of rough endoplasmic reticulum. Tendons and ligaments have a similar structure; however, some differences in responses of fibril populations to exercise may be explained by the higher cellularity and metabolic activity of ligaments.25

Results of the study reported here disproved our hypothesis that exercise would accelerate typical age-related reductions in collagen fibril MAD. However, we did document that there is age-related reduction in the equine CDET between 24 and 39 months of age. This is in agreement with limited data from other studies of the equine CDET, SDFT, and suspensory ligament. The mean fibril diameter in the calcaneus tendon of humans peaks at 18 years and then decreases with age;26 it therefore appears that reductions of fibril diameter in horses and humans is not restricted to those of advanced age but begins in young adults or adolescents, respectively. The decrease in MAD in the CDET in the study reported here was attributable to reduced numbers of fibrils $> 300$ nm in diameter and an increase in the numbers of fibrils $< 100$ nm in diameter in older horses. These changes were not accompanied by any alterations in the percentage area of the matrix occupied by collagen fibrils (ie, CFI). Age-related reductions in MAD may result from mechanical disruption of existing fibrils or altered direct or indirect cellular regulation of fibril assembly. Decreases in maximum stress with age have been correlated with reduced fibril diameters in tendons of other species.27
The apparent hypertrophy of the CDET of trained horses for the short-term exercise group was attributed to acceleration of an age-related phenomenon. A similar hypertrophy of digital extensor tendons without changes in flexor tendon cross-sectional area was documented in pigs subjected to a 12-month running regimen. Exercise-induced hypertrophy has been documented in the SDFT of equines in other studies, including 2- and 3-year-old Thoroughbreds during the first 4 months of race training and transiently in the SDFT of younger trained horses (2 to 15 months of age); the latter was also an acceleration of the rate of increase of cross-sectional area during maturation. Results of the study in which investigators used horses during the first months of race training were questionable because 2 of the trained horses developed clinical signs of mild tendonitis. However, in the short-term and long-term exercise groups in the study reported here, there was no evidence of SDFT hypertrophy; which is consistent with tendon-specific differences in mechanisms that control responses to mechanical loading.

The relationship between tendon hypertrophy and constituent collagen fibril populations remains uncertain. The manner in which the CDET increased in cross-sectional area in the young exercised horses could have had a bearing on the results of ultrastructural analysis, given the necessarily small area from which fibril measurements were obtained; to measure a minimum of 1,000 fibrils, we analyzed a total area of 62 to 82 μm²/specimen. Assuming tendons grow by expansion of existing fascicles, sites for collection of specimens for ultrastructural analysis within the fascicle may be of importance. New fibrils could be added in certain areas of the fascicle (eg, the periphery), or this may be a more uniform process whereby many or all existing tenocytes synthesize new fibrils. Cellular components and immediate pericellular regions were avoided in this and other studies, and because fibrillogenesis takes place in cytoplasmic recesses of tenocytes, this may have biased the measurements. It is also possible that new fibrils are only added in certain fascicles (eg, those in the periphery of the tendon), which were not analyzed. Sequential analysis of growing tendons would be required to understand the mechanisms by which there are increases in cross-sectional area; however, specimens for analysis of fibril diameter were only obtained at the termination of each exercise regimen. Biopsy specimens were not obtained from horses at various time points. In 1 preliminary study, horses had substantial tendon damage with reactive inflammation and scarring in response to a biopsy procedure. However, a study in human patients in clinical settings revealed the usefulness of the examination of tendon biopsy specimens, and it is possible that such an approach could be used in future equine exercise studies.

Additional explanations for the failure to observe changes in fibril MAD in the CDET could be that the exercise regimen was insufficient to induce such a change or that differences between amounts of exercise for the trained and control groups were not sufficient. We do not know how the amount of walking exercise for the control groups would compare with typical walking, trotting, cantering, and galloping exercise in horses in a pasture for that age group. However, increases in bone mineral density were measured in dorsal aspects of the carpal bones of exercised but not control horses from both exercise regimens, indicating that the training regimens were sufficient for a differential osteoinductive response. Also, in terms of the digital tendons, the long-term exercise regimen resulted in a reduction in MAD in the SDFT and the aforementioned CDET hypertrophy in the short-term exercise group. The differences in response of fibril populations in the 2 types of tendon could be explained by an inhibition or downregulation of the cellular reaction to increased loading specifically in energy-storing tendons because an increase in tendon cross-sectional area with constant frictional loads would decrease the peak strains and thus the amount of recoverable energy. This feature of energy-storing tendons, which results in low safety factors and reduced matrix turnover, in part explains the high prevalence of injury in these specific structures. The equine CDET maintains cellularity and collagen turnover with age, implying continued cellular activity in this positional tendon that could allow maintenance of collagen fibril populations even when subjected to an increase in loading. We are not aware of evidence that adaptive changes can develop and persist in tendons of adult horses. Assuming that certain training regimens could be used to stimulate such changes in clinically relevant tendons (eg, the SDFT), they would be of interest to trainers. It is possible that this may develop in horses younger than those used in the study reported here, which could provide a case for a controversial practice of initiating training in immature horses in addition to supporting racing at a younger age. Additional studies are required to determine whether certain training regimens can be developed to induce permanent adaptive responses in matrix structure in specific positional or energy-storing structures to maximize performance while minimizing injury, thus improving the welfare of racehorses.

References
3. Alexander RM. Springs as energy stores: running. In: Elastic

Appendix

Short-term and long-term training regimens for young female horses exercising on a treadmill.

<table>
<thead>
<tr>
<th>Day</th>
<th>Short term</th>
<th>Long term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monday</td>
<td>4.8 km at 10 m/s</td>
<td>3 km at 12 m/s</td>
</tr>
<tr>
<td>Tuesday</td>
<td>Trotting for 20 min</td>
<td>Trotting for 10 min</td>
</tr>
<tr>
<td>Wednesday</td>
<td>0.8 km at 13 m/s, 1 min of recovery, 0.8 km at 13 m/s, 1 min of recovery, and 0.8 km at 13 m/s</td>
<td>1.5 km at 12 m/s, 5 min of recovery and 1.5 km at 14 m/s</td>
</tr>
<tr>
<td>Thursday</td>
<td>Trotting for 20 min</td>
<td>Trotting for 10 min</td>
</tr>
<tr>
<td>Friday</td>
<td>1.3 km at 11 m/s, 2 min of recovery, and 1.3 km at 11 m/s</td>
<td>1 km at 12 m/s, 5 min of recovery and 1 km at 15 m/s</td>
</tr>
<tr>
<td>Saturday</td>
<td>Trotting for 10 min</td>
<td></td>
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</table>