Can exercise modulate the maturation of functionally different immature tendons in the horse?

Y. Kasashima, T. Takahashi, H. L. Birch, R. K. W. Smith, and A. E. Goodship

Equine Research Institute, Japan Racing Association, Utsunomiya, Japan; and The Royal Veterinary College and Institute of Orthopaedics and Musculoskeletal Science, University College London, London, United Kingdom

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Can exercise modulate the maturation of functionally different immature tendons in the horse? J Appl Physiol 104: 416–422, 2008. First published December 13, 2007; doi:10.1152/japplphysiol.00379.2007.—Tendons can be considered in two functional groups, those contributing to energetics of locomotion and those acting solely to position the limb. The energy-storing tendons in both human and equine athletes have a high frequency of injury with similar pathophysiology. In previous studies, high-intensity exercise appears to induce a disruption of the matrix rather than functional adaptation in adults. Here we explore the hypothesis that the introduction of controlled exercise during growth would result in an adaptive response without deleterious effects. Young horses were given a controlled exercise program similar to that previously shown to induce matrix changes in energy-storing tendons of skeletally mature animals. The tendons were assessed in relation to mechanical properties, molecular composition, and morphology. Results showed a significant increase in cartilage oligomeric matrix protein (COMP) in the positional tendon but not in the energy-storing tendon. Other matrix properties and mechanical properties were not significantly changed. While the imposition of high-strain-rate exercise in immature horses failed to augment the development of the energy-storing tendon over and above that induced by normal pasture exercise, it did not induce deleterious changes, supporting an earlier introduction of athletic training in horses.

adaptation; hypertrophy; cartilage oligomeric matrix protein; biomechanics

TENDON OVERUSE INJURIES are not only responsible for considerable loss of athletic potential in both horses (30, 35, 58) and humans (64), but their prevalence appears to be increasing, with a doubling in Achilles tendinopathy in recent years (26, 27, 34, 37, 38). While tendons that link muscle to bone can act merely as force transmitters to position limbs, a specific subgroup of tendons whose function is to store elastic energy contributes to efficient locomotion by acting as springs, while the associated muscle fibers serve primarily to dampen vibrations (1, 59). The Thoroughbred racehorse is a prime example of an elite animal athlete both in terms of evolution and subsequent genetic selection and conditioning. The superficial digital flexor tendon (SDFT) in the horse is an energy-storing tendon with functional and compositional similarities to the Achilles tendon in humans (7). This tendon is highly susceptible to injury, accounting for 93% of all tendon and ligament injuries in a study by Ely et al. (15) and thus can be used as a “natural” model to study tendon development and degeneration (51). The common digital extensor tendon (CDET) in contrast is a good example of a positional tendon analogous to the anterior tibialis in the human (3, 62).

Over the last two decades there has been a great increase in the scientific understanding of the response of bone and muscle to mechanical loading. The mechanobiology of tendon, however, is less well understood. Effects of exercise on tendon properties have been studied in various species, including mouse (36), rat (49, 55), rabbit (61), chicken (2), guinea fowl (8), miniature swine (60), and horse (5, 10–12, 40–42). In these studies the exercise regimens were defined in different ways, and both adaptive and degenerative effects have been reported. Differences in response may be related to a combination of factors, including age, species, functional input, and the specific tendon and anatomical site within the tendon studied. Interestingly, in the horse there appears to be a consistent absence or lack of functional hypertrophy in response to increased exercise in the tendons that act as elastic energy stores (5). Although this apparent lack of adaptive response may appear counterintuitive, these structures have optimized their mechanical properties to become finely tuned as energy-storing springs. Consequently, adaptive hypertrophy could reduce the levels of usable energy storage and thus compromise locomotor efficiency in an animal of the same weight and running at the same speed if the composition and material properties of the tissue remain the same. The mechanosensitivity of the cells from energy-storing tendons has also been shown to decrease with increasing age (22, 43).

There is convincing evidence that the injury in energy-storing tendons is associated with preceding degeneration characterized by structural and molecular changes within the tendon matrix, and this degeneration is related to both ageing and exercise (9, 48, 52). This observation has been supported by recent in vitro studies investigating the effects of repetitive loading on tendon matrix that show a loss of strength and upregulation of metalloprotease activity (13, 33). Collagen fibril diameters have been related to material properties of tendon tissue (6), and the fibril distribution is different between the different functional types of tendon (14). While the non-collagenous proteins make up only a small component of the total dry matter of the tendon, they have important roles in regulating matrix organization and function and, furthermore, are more labile component than collagen. Exercise in the skeletally mature animal resulted in a lower mass-average diameter of collagen fibrils without a change in total collagen content, a reduction in collagen crimp angle and length (40–42), and accelerated the age-related loss in COMP and glycos-
MATERIALS AND METHODS

controlled exercise during growth would result in an adaptive response that is potentially beneficial. Analyses were performed on the same horse postmortem at the end of the study (5, 29). We therefore hypothesize that during this process there is a capacity to exhibit functional adaptation. In support of this hypothesis, the changes in the dimension and mechanical properties of tendons after stress shielding have been shown to be greater in younger animals (20). Analogous to the situation in bone where only short episodes of loading are necessary to increase bone formation, appropriate short exercise regimes early in life will potentially improve the quality of the developing tendon, making it more able to withstand the later rigorous of subsequent athleticism and ageing (56).

We reported previously that imposed exercise in immature equine energy-storing tendon accelerated its growth in vivo but did not induce any significant difference in cross-sectional area between groups at the end of the study (5, 29). In this study, morphological, compositional, and biomechanical analyses were performed on the same horse postmortem at the end of the study to test the hypothesis that the early introduction of controlled exercise during growth would result in an adaptive response without deleterious effects on the matrix.

MATERIALS AND METHODS

Horses and Training Program

Fourteen Thoroughbred foals were divided randomly into the exercise and control groups (matched for sex; 5 female and 2 male). Weight and height were measured initially and at monthly intervals throughout the study. From birth the control group received pasture exercise for 4 h/day with the remainder of the day spent in a stable. The exercise group received the same amount of pasture activity but, in addition, received a period of controlled and defined treadmill exercise, daily for 5 days/wk, after the pasture exercise before returning to their stable overnight. A treadmill (Mustang, Fahrwangen, Switzerland) was used to impose a defined regime of high-speed gaits. After an initial warm up for 30 s at the walk and 1 min at trot, five 15-s periods of galloping were given with 30-s episodes of trotting interspersed. After the fifth gallop, 1 min of trot and 30 s of walk were given for cooling down. The trot speed was 2.5 m/s initially and 3.3 m/s from 150 days of age. Cantering speed was increased incrementally from 5 m/s at 60 days to reach 11 m/s by the end of the study (440 days). The activity program was carefully chosen to provide at least twice the maximal measured distance cantered by the control foals at pasture, and by giving this exercise immediately before the overnight rest it did not result in a compensatory decrease in pasture exercise levels in the exercise group. Canter distance was ~600 m/day at pasture and 1,400 m/day for the exercised group at the end of the study (17, 29).

Sampling Procedure and Sample Processing

All foals were euthanized at 15 mo of age in a humane manner. All animal protocols were approved by the Ethics Committee for Laboratory Animals of Japan Racing Association Equine Research Institute. Left and right forelimbs were removed at the scapulohumeral joint, and the skin was removed. The level of the midpoint of the third metacarpal bone was marked on both the SDFT and the CDET with the limb held in the stance position. Tendons were inspected visually for gross lesions and were dissected from the limb.

The SDFT and CDET from the left limb were double wrapped in cellophane and stored frozen at −20°C for mechanical testing as described below. A 2-cm-long sample of the SDFT and CDET from the right forelimbs, taken from the midmetacarpal region, was snap frozen in an isopentane bath on dry ice and stored at −80°C for molecular composition analysis. A 0.5-cm section proximal to this sample was used for electron microscopy. Strips 1 × 1 × 5 mm³ were dissected from both the SDFT and CDET, which were fixed in 2% paraformaldehyde (Merck) and 2.5% glutaraldehyde (Taab) in 0.1 M cacodyl buffer at pH 7.4 for 5 h at 4°C.

Ultrastructural Analysis

Sample strips described above were treated with 1% osmium oxide for 2 h at room temperature, washed in distilled water, and dehydrated first in a graded ethanol series (70%, 90%, 95%, and 100%; 3 times successively) and then three times in 100% butyl glycidyl ether (QY-1, Oken, Japan). Samples were immersed in a 50:50 solution of butyl glycidyl ether and resin (Epok 812; Oken), which was then replaced with 100% resin twice before resin embedding. Thick sections (90–130 µm) were cut from one block for each tendon region using a diamond knife, mounted on copper grids and stained with 2.5% uranyl acetate followed by 5% phosphotungstic acid.

The sections were viewed using a Philips CM12 high-resolution electron microscope. One representative section was examined from each SDFT and CDET. Ten to fifteen micrographs were taken randomly from different areas of each section, which was sufficient to allow analysis of at least 1.000 collagen fibril diameters in CDET and 1,800 in SDFT (39). All magnifications were ×68,000. The analysis program (NIH image, National Institutes of Health Research Services Branch, Washington, DC) required uniform staining of fibrils and adequate contrast between fibrils and extracellular matrix. To achieve this, the outline of each collagen fibril was traced onto a sheet of tracing paper set on the micrograph. The traced outlines were digitized and used in the image analysis program to determine the diameters of the fibrils. Where fibrils had not been cut transversely, the short axis was determined, which is representative of the transverse diameter. The total area of each image selected for analysis in the micrograph was also recorded, and the percentage of each image area occupied by collagen fibrils calculated, giving the collagen fibril index (CFI) (16).

A diameter-frequency distribution was calculated from these data and entered into a computer program developed by Parry (41). This program uses a calibration factor to convert the diameter measurements in millimeters to nanometers and then calculates the percentage of total measured fibril area occupied by each diameter group. From this, the mass-average diameter (MAD) was determined. This provides a more accurate analysis of fibril-related area as it takes into account the fact that usually small numbers of large-diameter fibrils occupy a large proportion of the cross-sectional area. The MAD of a collagen fibril population is derived from the fibril diameters as a function of the percentage of fibril area.

Molecular Compositional Analyses

Water content. Approximately 900–1,700 mg of each tendon sample was weighed out accurately on a digital balance with precision to 0.01 mg. Tissue was refrozen at −80°C and freeze-dried under vacuum until a constant weight was reached. Lyophilized tissue was then reweighed to give a dry weight. Water content was expressed as a percentage of the wet weight.

GAG assay. Following water content measurements, lyophilized tissue was powdered in a mixer mill (MM200, Retsch), and aliquots were prepared in 1 M hydrochloric acid (HCl) for analysis.

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(−20 mg accurately weighed) were suspended in 2 ml sterile PBS, pH 6.0, containing 5 mM cysteine HCl and 5 mM EDTA. Papain (16–40 U activity/mg) was then added at a concentration of 125 µg/ml, and digestion was carried out at 60°C for 24 h.

Total sulfated GAG content was quantified from the solubilized extract using dimethylmethylene blue dye (18). Concentrations were calculated by comparison with a standard curve prepared with purified bovine trachea chondroitin sulfate (0–10 µg in 3 ml dye). Results were expressed as micrograms chondroitin sulfate-equivalent sulfated GAG per milligram dry weight tissue.

Collagen content. Collagen content was calculated indirectly by measuring the imino acid hydroxyproline. One-hundred microliters of papain digest were hydrolyzed with in 6 M HCl at 110°C for 24 h. Samples were dried under vacuum, and the residue was dissolved in deionized water. Hydroxyproline was assayed using Ehrlich’s reagent by a method developed from that of Bergman and Loxley (4). Briefly, the sample solutions were oxidized with chloroamine T and reacted with the Ehrlich’s color reagent (4-dimethylaminobenzaldehyde). The absorbance was measured at 550 nm in a spectrophotometer. Hydroxyproline concentrations were calculated by comparison with a standard curve prepared with L-hydroxyproline standards (1–10 µg hydroxyproline/ml), and collagen content was calculated assuming hydroxyproline to be present at 14% in collagen. Collagen content was expressed as a percentage of the tissue dry weight.

COMP assay. Lyophilized tissue (50 mg) was extracted using 80 volumes of 4 M guanidine hydrochloride including 5 mM N-ethylmaleimide and protease inhibitors. The supernatant was analyzed for COMP using a homologous inhibition ELISA as described previously using purified equine COMP as coating and standards, and an equine specific polyclonal antiserum against equine COMP (53). COMP content was expressed as micrograms per milligram wet weight tissue.

Mechanical Property Analyses

Tendons were thawed at room temperature, and loose connective tissue was removed from the surface of the tendon. The cross-sectional area was measured at the midmetacarpal level of the left and right limb tendons, using an aqueous-based precision dental impression material (Cremix, Dentsply) (23).

Tendons were mounted with the metacarpal region of the tendon equidistant between the two cryoclamps to give a gauge length of 100 mm (distance between the clamps) The proximal and distal ends of the tendon were secured in the clamps by freezing with liquid CO2 (44). Tendons were preloaded, to remove slack, with a load predicted to be ~1% of the failure load. (100 N for the SDFT and 25 N for the CDET), and the distance between the freeze lines was measured. Tendons were then preconditioned with 20 sinusoidal cycles to a load of 4.5 and 1.5 kN for the SDFT and CDET, respectively, at a frequency of 0.5 Hz. The function of these preconditioning cycles was to eliminate much of the viscous components of the viscoelasticity (46) and resulted in a steady load-deformation curve for all tendons. The tendons were then loaded to failure at a strain rate of 80 mm/s. The force and stroke (distance moved by the clamps) data were collected and used to calculate ultimate force, ultimate stress, ultimate strain, and from the linear portion of the load-deformation curve, stiffness and elastic modulus. Modulus was calculated from the stress (force/cross-sectional area) divided by strain (change in length/original length) calculated from the linear elastic region of the load-deformation plot.

Statistical Analysis

All statistical analyses were performed with commercially available software (Statview, SAS). All values were subjected to two-way ANOVA with Tukey-Kramer as a post hoc test to determine the effect of exercise (control and exercise groups) and tendon (SDFT and CDET). The level of significance was set at P < 0.05. Comparisons of the effects of exercise on each tendon were performed with unpaired t-test independently, since the data were distributed normally. In addition, percentiles (P0, P25, P50, P75, and P100) of the collagen fibril population were calculated and compared between each tendon by using paired t-test.

RESULTS

Horses

The exercise and control groups matched for sex were not significantly different in weight and height throughout the study.

Tendon Cross-Sectional Areas

No significant differences were found in tendon cross-sectional areas between groups for either SDFT or CDET (see Fig. 3).

Collagen Fibril Morphology

The fibril diameter distribution for the SDFT and for the CDET in each group is represented graphically in Fig. 1. There was no significant difference in percentile values for tendon between the exercise and control groups. The percentile values obtained from the CDET were significantly larger than those of SDFT (P < 0.05, Table 1).

The MAD and CFI were significantly higher in the CDET than in the SDFT for exercise and control groups (P < 0.05). However, no significant differences were seen in the MAD and CFI between the exercise and control groups for the SDFT and the CDET.

![Fig. 1. Comparison of collagen fibril diameter distributions between exercise (n = 7) and control (n = 7) groups. SDFT, superficial digital extensor tendon; CDET, common digital extensor tendon.](image-url)
Molecular Composition

The CDET had a significantly lower mean water content of 59.1 ± 0.6% (values are means ± SD) than the SDFT (70.1 ± 2.3%) (P < 0.05) but was not significantly different between exercise and control tendons for either the SDFT or the CDET. The mean total sulfated GAG content of the CDET (3.91 ± 0.29 μg/mg dry weight) was significantly (P < 0.05) lower than the SDFT 14.05 ± 3.46 μg/mg dry weight. No significant differences in GAG content were seen between exercise and control groups for the SDFT and the CDET. The mean total collagen content was 77.5 ± 2.8% of the dry weight in the SDFT and 85.1 ± 2.1% in the CDET, which was significantly different (P < 0.05). There were no significant differences between exercise and control groups of tendons.

The mean COMP content of the CDET in the controls (2.2 ± 0.8 μg/mg dry weight) was significantly lower than the overall mean value of the SDFT (10.2 ± 3.3 μg/mg dry weight). No significant difference in COMP content was seen between exercise and control groups for the SDFT. However, the COMP content in the CDET was significantly (P = 0.0419) higher in the exercise group of tendons compared with the control tendons (Fig. 2 and Table 1).

Mechanical Properties

The structural properties, ultimate force and stiffness, were significantly higher for the SDFT compared with the CDET; however, the ultimate stress and elastic modulus, which are material properties, were significantly higher for the CDET compared with the SDFT (Figs. 3 and 4). Ultimate strain values were not significantly different between the flexor and extensor tendons, and there were no differences in any of the material and structural properties between exercise and control groups.

DISCUSSION

The additional exercise given in this controlled study in immature horses did not induce changes in the mechanical properties or the major matrix components of the energy-storing SDFT and only altered one of the measured matrix components in the CDET. However, no deleterious effects were seen, in contrast to those observed in similar studies in adult horses.

Table 1. Summary of SDFT and CDET composition in comparison in exercise and control groups of foals

<table>
<thead>
<tr>
<th>Component</th>
<th>SDFT</th>
<th>CDET</th>
<th>Mean*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>Control</td>
<td>Exercise</td>
<td>Control</td>
</tr>
<tr>
<td>MAD, nm</td>
<td>142.7±15.6</td>
<td>156.4±22.8</td>
<td>149.5±20.0</td>
</tr>
<tr>
<td>Collagen fibrillar index, %</td>
<td>66.3±5.5</td>
<td>68.0±6.9</td>
<td>67.2±6.1</td>
</tr>
<tr>
<td>P0, nm</td>
<td>19.0±2.6</td>
<td>21.2±2.6</td>
<td>20.1±2.7</td>
</tr>
<tr>
<td>P25, nm</td>
<td>38.4±4.8</td>
<td>40.2±4.2</td>
<td>39.3±4.3</td>
</tr>
<tr>
<td>P50, nm</td>
<td>48.7±7.1</td>
<td>49.9±6.6</td>
<td>49.3±6.7</td>
</tr>
<tr>
<td>P75, nm</td>
<td>79.3±30.4</td>
<td>87.7±37.9</td>
<td>83.5±33.3</td>
</tr>
<tr>
<td>P100, nm</td>
<td>253.4±10.9</td>
<td>270.7±27.7</td>
<td>262.1±22.2</td>
</tr>
<tr>
<td>% Water content</td>
<td>69.7±2.29</td>
<td>70.35±2.49</td>
<td>70.05±2.32</td>
</tr>
<tr>
<td>GAG, μg/mg</td>
<td>13.73±3.91</td>
<td>14.37±3.22</td>
<td>14.05±3.46</td>
</tr>
<tr>
<td>% Collagen content</td>
<td>78.02±1.79</td>
<td>76.90±3.57</td>
<td>77.46±2.77</td>
</tr>
<tr>
<td>COMP, μg/mg</td>
<td>10.41±3.66</td>
<td>10.01±3.27</td>
<td>10.21±3.34</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Mean refers to overall mean value of the superficial digital flexor tendon (SDFT) or common digital extensor tendon (CDET). MAD, mass-average diameter; P0, P25, P50, P75, and P100 are percentiles of the collagen fibril population; GAG, glycosaminoglycan; COMP, cartilage oligomeric matrix protein. †Significant difference compared with the SDFT. ‡Significant difference between exercise and control group in CDET.

The exercise level chosen was based on previous studies using skeletally mature horses in which the controlled exercise at a high-speed gait induced changes within the matrix of the core of the SDFT (41). However, to allow for the training and growth of foals, the exercise program was increased incrementally but at a level that was additional to the measured quantity of exercise undertaken naturally. The exercise input at the end of this study comprised a defined period of treadmill high-speed exercise superimposed on the measured level of free pasture exercise. This provided 8 km/wk of maximal speed gait; this compares with 10 km/wk in our previous study with older adult horses.

In contrast to the induced matrix changes seen in the adult horses, results of this study show remarkable similarities in the biomechanical properties and molecular composition between the two groups despite the specific exercise regimen imposing twice the volume of physiological high-loading activity of the
control foals (29). The most likely explanation for the accommodation of these levels of exercise with no adverse changes in the mechanobiology is that full adaptation occurred in response to a minimum number of loading cycles that was achieved in both groups. It is known that bone mass can be maintained, and increased, with only a very few loading cycles per day. Above a certain number of loading cycles, no further increase in bone mass was found (45). If this was the case, then pasture exercise, where cantering occurred and was measured by observation, may be expected to provide sufficient tendon loading cycles of sufficient magnitude to induce full adaptation, and thus additional imposed exercise would not induce any further adaptation. This concept is supported by data in a different study where differences in matrix were seen in foals severely restricted in exercise by box rest compared with foals undertaking natural pasture activity (10–12).

The specific characteristics of stimuli that actually provoke tendon adaptation have not been fully elucidated, but, by analogy to bone, cyclical high-strain-rate deformation of the tissue would be considered to have the greatest stimulatory potential. Strain rates in equine digital flexor tendon are related to the gait and the speed of the horse with the highest strain rates seen at the canter (54). These animals were cantering at 11 m/s (40 km/h) from 12–15 mo of age, which is considered a fast speed, even in the adult Thoroughbred, although the duration of this fast speed was brief. An alternative explanation that the exercise level may have been insufficient in amount or nature to provoke adaptation is less likely because thin-slice radiography of carpal bones from these horses showed adaptive changes to be present within individuals of both groups (data not shown). Rapid adaptation of this same bone has been reported previously in treadmill exercise studies, where treadmill exercise induced maximal site-specific increased bone density after only 4.5 mo of exercise (19). Furthermore, the imposition of the defined increased functional demand in these foals did induce a change in muscle using in vivo muscle biopsies (17). The exercise group was found to have hypertrophy of all muscle fiber types, and both type I and IIa were significantly larger by 12 mo of age. A significant increase of succinic dehydrogenase activity was also found in type IIx fibers in the exercise group (17). Our data also show a significant difference in matrix COMP levels in the positional CDET as a consequence of exercise. However, different levels of exercise may be needed to induce beneficial adaptations in energy-storing tendons.

It is also possible that the nature of adaptation was different from that analyzed. At a tissue level probably the most important effects would be to generate a matrix that is either able to repair microdamage more effectively than appears to be currently possible in adult tendon and/or a structure that is more resistant to fatigue damage. Exercise may have induced an

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**Fig. 3.** Structural properties of SDFT and CDET. *Significant difference ($P < 0.05$). Solid bracket refers to comparison between SDFT and CDET. Dashed brackets refer to comparison between exercise and control group.

**Fig. 4.** Material properties of SDFT and CDET. *Significant difference ($P < 0.05$). Solid bracket refers to comparison between SDFT and CDET. Dashed bracket refers to comparison between exercise and control group.
increased capacity of tenocytes to repair microdamage, but no analysis was performed on the metabolic activity of the cells within the tendon in this study. In addition, fatigue resistance is a difficult characteristic to measure objectively with biological relevance, although some attempts have been made in other species (31, 47, 63).

In contrast to the SDFT, the CDET showed a significant change in COMP levels in the trained group of horses. Other studies have also found a tendon-specific response to exercise. Positional tendons such as the CDET appear to undergo adaptive hypertrophy, while energy-storing tendons such as the SDFT do not (5, 60, 62). This finding is in keeping with other studies investigating the effects of exercise on the functionally similar adult human Achilles tendons (25, 32). The COMP levels were significantly higher in the CDET in the trained group of horses than in the controls. Although there were no significant differences in other parameters, there was a strong correlation ($r = 0.62$) between ultimate tensile strength and COMP levels (data not shown). Current opinion is that the role of COMP is one of matrix organization rather than directly contributing to mechanical strength (24, 51). Thus the findings in this study would be in keeping with this concept.

While the imposition of high-strain-rate exercise in immature horse did not induce deleterious changes to the functional morphology of the energy-storing tendon, as is the case in mature horses, it failed to alter the energy-storing capacity of the SDFT over and above levels induced by normal pasture exercise. Human athletes begin training early in life, while equine athletes frequently begin training relatively later in life. This study provides evidence that imposing additional exercise in the immature skeletal system is not deleterious but may bestow benefits for future athleticism and justifies an earlier introduction to training in horses.

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