Functional adaptation through changes in regional biochemical characteristics during maturation of equine superficial digital flexor tendons

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Objective—To quantify and compare biochemical characteristics of the extracellular matrix (ECM) of specimens harvested from tensional and compressive regions of the superficial digital flexor tendon (SDFT) of horses in age classes that include neonates to mature horses.

Sample Population—Tendon specimens were collected on postmortem examination from 40 juvenile horses (0, 5, 12, and 36 months old) without macroscopically visible signs of tendonitis.

Procedure—Central core specimens of the SDFT were obtained with a 4-mm-diameter biopsy punch from 2 loaded sites, the central part of the mid-metacarpal region and the central part of the mid-sesamoid region. Biochemical characteristics of the collagenous ECM content (ie, collagen, hydroxylysylpyridinoline crosslink, and pentosidine crosslink concentrations and percentage of degraded collagen) and noncollagenous ECM content (percentage of water and glycosaminoglycans, DNA, and hyaluronic acid concentrations) were measured.

Results—The biochemical composition of equine SDFT was not homogeneous at birth with respect to DNA, glycosaminoglycans, and pentosidine concentrations. For most biochemical variables, the amounts present at birth were dissimilar to those found in mature horses. Fast and substantial changes in all components of the matrix occurred in the period of growth and development after birth.

Conclusions and Clinical Relevance—Unlike cartilage, tendon tissue is not biochemically blank (ie, homogeneous) at birth. However, a process of functional adaptation occurs during maturation that changes the composition of equine SDFT from birth to maturity. Understanding of the maturation process of the juvenile equine SDFT may be useful in developing exercise programs that minimize tendon injuries later in life that result from overuse. (*Am J Vet Res* 2005;66:1623–1629)

Horses are precocious animals, and the typical foal stands 55 minutes after birth.[1] Therefore, in horses, birth signifies a dramatic physiologic change not only for the respiratory and cardiovascular systems but for the musculoskeletal system as well. Immediately after birth, the musculoskeletal system is faced with new postural, locomotive, and gravitational forces. Tissues that make up the musculoskeletal system are in a phase of rapid growth and adaptation and are able to readily adapt to these changes.[2] The study of these adaptive processes in various tissues will yield valuable information that may have implications for the maximum exercise load of young horses.

In articular cartilage, it has been shown that a loading-driven process of functional adaptation exists at an early age, shaping the biochemical make-up of the tissues.[3] In this process, a change occurs from a homogeneous blank joint to a distinct topographic heterogeneity with respect to all constituents (collagenous and noncollagenous) of the **extracellular matrix (ECM)**.[4,5] This heterogeneity in biochemical characteristics matches the biomechanical challenge and seems to be formed under the influence of early postnatal biomechanical loading in a so-called process of functional adaptation.[3,6] This is not unlike the adaptation of bone to loading as already described by Julius Wolff in the late 1800s.[7]

Tendon tissue and articular cartilage have several features in common. They have a large ECM-to-cell ratio and consist of basically the same following components: collagen fibrils, proteoglycan aggregates, and water. As in cartilage, the biochemical composition varies considerably between regions, apparently reflecting differences in loading.[8] These similarities may lead to the presumption that in tendons, the same concept of gradual functional adaptation applies.

Research into the development of tissue characteristics of tendons in juvenile horses has been limited. Work has mainly focused on ultrastructure and biomechanical characteristics. From this work, a picture of rapid and profound changes emerges. At birth, the equine **superficial digital flexor tendon (SDFT)** is highly cellular with a so-called unimodal distribution of small collagen fibrils and a high proportion of type III collagen.[9,10] During postnatal development, fibril distribution changes into a bimodal one[11] and the collagen type changes to a clear predominance of type I. With maturation, biomechanical characteristics of the SDFT, such as elastic modulus,[12] crimp angle and crimp length,[13] collagen fibril mass-average diameter, and collagen fibril index,[14] change gradually, resulting in an overall increase of tendon stiffness and strength.[a] The purpose of the study reported here was to quantify and compare biochemical characteristics of the ECM

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of biopsy specimens harvested from tensional and compressive regions of the SDFT of horses in age classes that included neonates to mature horses. We hypothesized that the biochemical composition of the tendon from the metacarpal and sesamoid regions would be identical at birth but would become distinctly different as horses matured.

Materials and Methods

Specimen collection—Tendon specimens were collected on postmortem examination from 40 juvenile horses, age 0 (n = 8), 5 (8), 12 (8), and 36 (8) months and mature horses (n = 60; mean ± SD age, 12.2 ± 4.6 years; range, 4 to 23 years). The foals aged 0 months were stillborn foals or foals that died at delivery, the juvenile horses aged 5 to 36 months were euthanatized for other reasons than orthopaedic ailments by use of an overdose of barbiturates[b] after sedation with detomidine[c] (0.1 mL/100 kg), and the mature specimens came from slaughter horses. All horses were warmbloods, and tendons had no visible signs of any tendinopathy. Specimens were obtained with a 4-mm-diameter biopsy punch from the central part of the midmetacarpal region of the SDFT in all horses and from the central part of the midsesamoid region in all horses except for the 5-month-old horses. The specimens were split in half (in a dorsopalmar direction) to be used for the analysis of **glycosaminoglycans (GAGs)**, DNA, and **hyaluronic acid (HA)** concentrations and for collagen network analysis, respectively. After recording of wet weight immediately after specimen collection, specimens were lyophilized for 24 hours and dry weight was recorded. Specimens were frozen and stored at –80°C until further analysis.

Determination of water, DNA, GAG, and HA content—The percentage of water was calculated by use of the following equation: ([wet weight – dry weight]/wet weight) × 100%. Without preprocessing, tendon specimens (< 20 µg) for DNA, GAG, and HA analysis were digested by papain (1 U/mL)[d] in 400 µL of 50mM of phosphate buffer (pH, 6.5), containing 2.0mM Na_2EDTA and 2mM cysteine for approximately 18 hours at 65°C.[15] The DNA analysis was according to Kim et al.[16] Briefly, the fluorescent dye Hoechst 33,258 was added to papain-digested tendon, and fluorescence was measured immediately after mixing by use of a fluorimeter,[e] with excitation at 366 nm and emission at 442 nm. Calf thymus DNA[d] was used as a reference. Results were expressed as micrograms of DNA per milligram of dry weight. The GAG concentration was analyzed by use of the modified 1,9-dimethylmethylene blue dye[d] binding assay as described by Farndale et al[17] and measured by a microtiter plate reader[f] with an absorbency of 525 nm. Shark chondroitin sulfate[d] was used as a standard. Results were expressed as micrograms of GAG per milligram of dry weight. The HA concentration was measured by use of the microtiter plate assay as described by van den Hoogen et al.[18] Briefly, HA was measured after removing sulfated GAGs in a papain digest of tendons by adding an equal volume of a suspension of diethylaminoethyl sepharose[g] in 1M NaCl. The uronic acid residues in the supernatant were assumed to originate from HA and were quantified by a microtiter plate reader[f] at an absorbency of 525 nm. The change in absorbance reflects uronic acid, and values were standardized with galacturonic acid.

Determination of degraded collagen—An assay that is based on the observation that α-chymotrypsin digests denatured collagen, but not the intact triple helix of collagen type I, was used.[19] In brief, after treatment with α-chymotrypsin, the supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen) and both were hydrolysed in 6M HCl. The amount of the collagen-specific amino acid (hydroxyproline) released by α-chymotrypsin was determined after reaction with chloramine T and dimethylaminobenzaldehyde by absorption at 570 nm as described previously.[20,h] The percentage of degraded collagen was calculated from the amount of hydroxyproline by the following equation: [(hydroxyproline concentration in the supernatant α-chymotrypsin digest)/(hydroxyproline concentration in the supernatant α-chymotrypsin digest + hydroxyproline concentration of digested tendon plug)] × 100%.

Determination of total collagen concentration and crosslink analysis—Papain-digested tendon specimens were hydrolyzed in 6N HCl (110°C, 20 hours). After drying, the hydrolysates were dissolved in internal standard solution (10mM pyridoxine[d] and 2.4mM homoarginine[d] in water). For crosslink analysis, (**hydroxylysylpyridinoline [HP]** and pentosidine), samples were diluted 5-fold in 10% acetonitrile and 1% heptafluorobutyric acid solution[i] and crosslinks were quantified by reversed phase–high-pressure liquid chromatography.[21] For amino acid composition, these diluted samples were additionally diluted 50-fold with 0.1M borate buffer (pH, 11.4) and amino acids were derivatized by use of 9-fluorenylmethyl chloroformate.[i] The labeled amino acids were separated and quantified by reverse phase–high-pressure liquid chromatography as described previously.[22] Concentrations of crosslinks were expressed per collagen triple helix (mol/mol of collagen)[23] to normalize for variations in total collagen concentration. In all instances, 1 collagen molecule (triple helix) is assumed to contain 300 hydroxyproline residues. Collagen concentration was expressed as milligrams of collagen per milligram of dry-weight tendon (mg/mg of dry weight).

Data presentation and analysis—All measured biochemical variables were expressed as mean ± SD. Biochemical data were analyzed by use of a software program.[j] Normality was assessed by use of the Kolmogorov-Smirnov method, and data were found to be normally distributed, allowing the use of parametric tests. Differences between 2 regions and among age groups (different time points) were tested by use of a 2-way ANOVA. The Fisher least significant difference multiple comparisons test of the means was applied as a post hoc test. The level of significance was set at values of $P < 0.05$. Data from mature horses were obtained from a previous study.[8]

Results

Neonatal horses—In neonatal horses, no regional differences in the amount of water, HA, degraded collagen, and HP crosslinks were found (**Figures 1–4**). However, regional differences in DNA, GAG, and pentosidine concentrations were found that were significantly higher in the sesamoid region, compared with the metacarpal region (**Figures 5–7**). A regional difference in total collagen concentration was found that was significantly higher in the metacarpal region, compared with the sesamoid region (**Figure 8**).

Development during maturation—All biochemical variables significantly changed during the development from neonate to adult. Most biochemical variables had significant site differences at 1 or more stages during development, but the patterns along which these differences developed varied among variables.

Noncollagenous biochemical variables—The percentage of water was approximately 80% in both

regions at birth and then decreased gradually. This decrease was similar in both regions until 36 months of age, when mean percentage of water was 70%. From 36 months to maturity, a significant site difference developed with a higher percentage of water in the metacarpal region (63%) than in the sesamoid region (57%; Figure 1). The DNA concentration decreased after birth in both regions and thereafter stayed at the same concentration (sesamoid region) or increased again (metacarpal region). The DNA concentration was significantly higher in the sesamoid region at birth and at 12 months, compared with the metacarpal region. In mature horses, DNA concentrations were higher in the metacarpal region, compared with the sesamoid region (Figure 5). Total GAG concentration increased steadily but slowly in the sesamoid region throughout maturation. In the metacarpal region, a steep increase in GAG concentration was found during the first 5 months, followed by a sharp decrease thereafter. The GAG concentration was significantly higher in the sesamoid region at birth and in mature horses, compared with the metacarpal region. The GAG concentration was significantly higher in the metacarpal region at the age of 12 months, compared with the sesamoid region (Figure 6). The HA concentrations increased rapidly and significantly from birth until 5 months in both regions. In the metacarpal region, the HA concentrations decreased afterwards. In the sesamoid region, the increase continued until 12 months of age, and then HA concentrations started to decrease until 36 months of age, after which a small increase was observed again. Concentrations were higher in the sesamoid region in the 12-month-olds and in mature horses, compared with the metacarpal region (Figure 2).

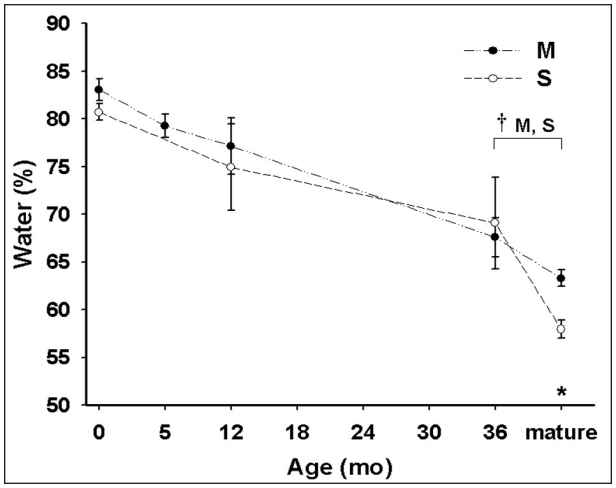

Figure 1—Mean ± SD percentage of water, as a noncollagenous extracellular matrix component, versus age in normal equine superficial digital flexor tendon (SDFT) tissues from 2 differently loaded sites. M = Tissue biopsy specimens from the central core of the metacarpal region of SDFT. S = Tissue biopsy specimens from the central core of the sesamoid region of SDFT. *Significant ($P < 0.05$) difference between regions. †Significant ($P < 0.05$) difference between age groups in either the M or S region.

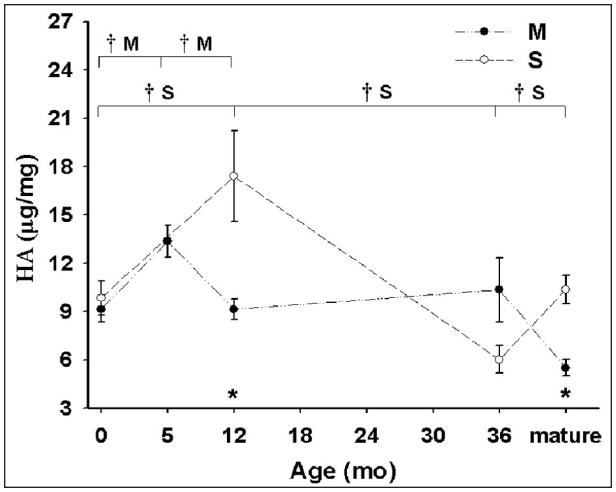

Figure 2—Mean ± SD concentration of hyaluronic acid (HA) on a dry-weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

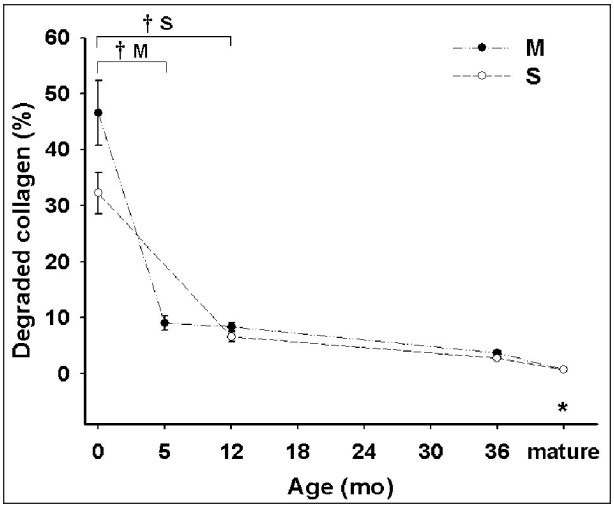

Figure 3—Mean ± SD percentage of degraded collagen, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

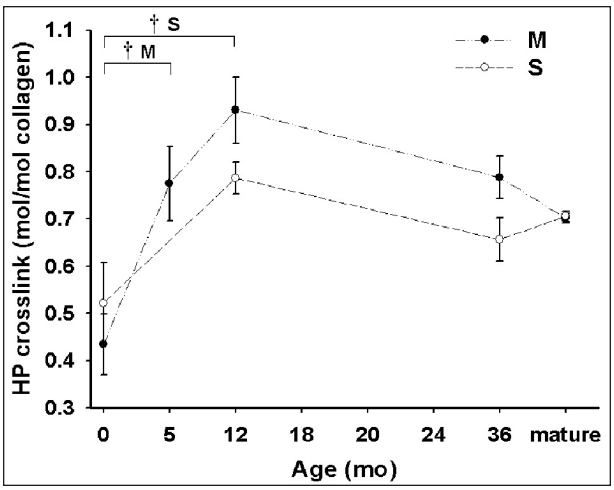

Figure 4—Mean ± SD concentration of hydroxylysylpyridinoline (HP) crosslinks, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

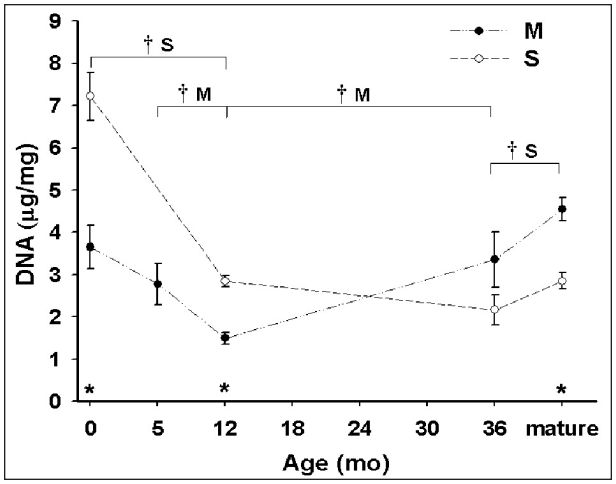

Figure 5—Mean ± SD concentration of DNA on a dry-weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

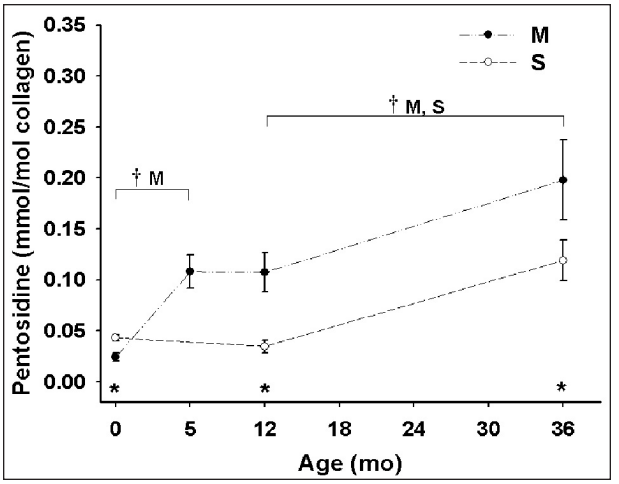

Figure 7—Mean ± SD concentration of pentosidine crosslinks, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

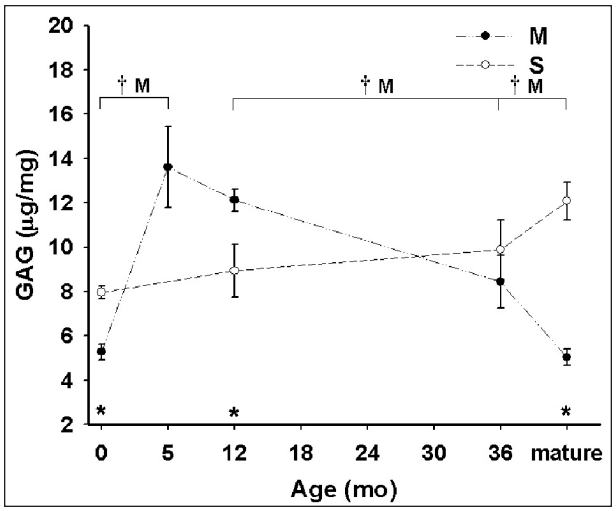

Figure 6—Mean ± SD concentration of glycosaminoglycan (GAG) on a dry-weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

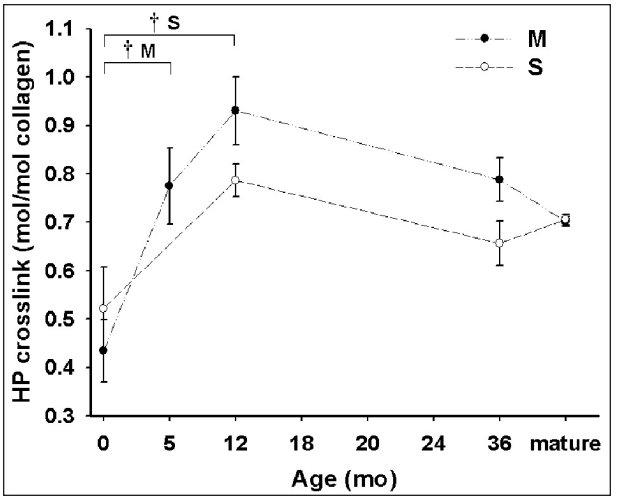

Figure 8—Mean ± SD concentration of total collagen on a dry-weight basis, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

Collagen biochemical variables—Collagen concentration decreased from birth to 5 months of age in the metacarpal region. After this, the collagen concentration in the metacarpal region remained constant until 36 months of age, followed by an increase. In the sesamoid region, collagen concentrations slowly decreased throughout the entire period. Collagen concentration was significantly higher in the metacarpal region at birth and in mature horses (Figure 8). The percentage of degraded collagen was similar between the 2 sites. A sharp decrease after birth occurred until approximately 5 months of age, and then a steady state was reached. The relative amount of degraded collagen in mature horses was approximately 3% (Figure 3). The HP crosslink concentrations increased significantly during the first 12 months of life and then reached a steady state. No significant difference in HP crosslink concentration was found between the 2 sites (Figure 4). Pentosidine crosslink concentrations were higher in the sesamoid region than in the metacarpal region at birth. After that, a gradual increase was found in both regions, and at 36 months of age, pentosidine concentrations were significantly higher in the metacarpal region, compared with the sesamoid region (Figure 7). In an earlier study,[8] pentosidine concentrations in the mature horses continued to increase in the sesamoid region (but not in the metacarpal region); therefore, mean pentosidine concentrations were not given for mature horses.

Discussion

In newborn horses, the composition of the ECM at the 2 differently loaded sites was dissimilar with respect to some measured biochemical variables. Therefore, unlike in articular cartilage, already some topographic heterogeneity exists at birth in tendon tissue, and foals are hence not born with a blank (homogeneous) tendon. Although other factors cannot be

excluded, movements of the fetus in utero, which have been determined to be a prerequisite for the correct development of functional tendons,[24] may be a cause. Fetal movements influence the expression in tendon tissue of vascular endothelial growth factor, which is one of the most important angiogenic factors during embryo genesis.[25,26] Articular cartilage may be considered as unloaded during fetal development, but movements of the fetus in utero will exert some forces on the developing tissue of the flexor tendons. These forces will be principally tensional; substantial compressive forces will not be exerted until the foal is on its feet and subjected to gravitational forces. However, the pulley-like conformation of the SDFT in the sesamoid region will lead to different force patterns at that site than in the metacarpal region where the force vector is exactly in line with the long axis of the tendon.

The connective tissues of the musculoskeletal system develop from a relatively simple mesenchymal ECM in the embryo.[27] In mature horses, this original tissue has differentiated into various specialized tissue types that are adapted to withstand various forms of mechanical stress such as compression, tension, and shear. This extensive remodeling is thought to be effected through the mechanical stimulation of integrins (or associated proteins), which trigger adaptive cellular responses.[28,29]

Biomechanical challenges to the equine flexor tendons change during the development from fetus to mature horse. Various periods can be discerned. Movements take place in utero but without the influence of gravitational forces. An abrupt change occurs after birth when the structures are first subject to gravity and concomitant weight bearing. In the immediate postnatal period, rapid growth occurs and thus a rapid increase in weight bearing. The onset of locomotive activities will further increase the mechanical challenge of the tissues in the period immediately after birth. After these first months, growth slows down and locomotion will remain approximately the same. Around 3 years of age, the typical warmblood horse will be taken into training, and hence athletic challenge will increase. Once the horse is in full athletic activity, the mechanical environment will not change much during the rest of the horse's functional life. It can be assumed that these variations in mechanical stimulation will have their effect on the connective tissues of the musculoskeletal system.

To a limited extent in our study, interference by batch differences in the biochemical analyses may have occurred, as it was not possible to analyze the large number of samples in a single batch. This is a potential weakness of our study. However, standards were added in each batch for analysis of DNA, GAG, and HA, and the samples were run in triplicate. For the collagen biochemical variables, internal standards were always used for each run by use of high-pressure liquid chromatography. During analyses, cross-reference checks were performed that revealed that almost no difference occurred in the DNA, HA, and dimethylmethylene blue assays (interassay coefficient of variation was below 5% in all assays). The current routine laboratory procedures have been proven to yield repeatable results in earlier studies,[3,15,30] and it was felt that group comparisons were possible.

In the development of various biochemical components from birth to maturity, some general patterns emerged. A general pattern was found in the percentages of water and degraded collagen and in HP crosslink concentration, where the biochemical variables start at similar amounts at both investigated sites followed by age-related changes in these variables that are similar at both sites. To a large extent, the same applies to pentosidine concentrations, although a small, but significant, difference occurs in pentosidine concentrations between the 2 sites in neonatal horses.

The percentage of water decreased from 80% at birth to approximately 65% at age 3 years in an almost linear fashion, indicating that the desiccation of the tissue is a time-related process that is apparently not affected by the biomechanical environment. The high percentage of water in the metacarpal region in mature horses has been linked to the accumulation of microdamage[8] and may hence be degenerative in character.

The relative amount of degraded collagen decreases rapidly during the first 5 months of life and levels out afterwards. The assay cannot discriminate between degraded collagen generated by physiologic turnover or by pathologic processes. However, this quick decrease in the percentage of degraded collagen during the first months of life in healthy foals seems indicative of the decrease in remodeling rate of structural elements of the tendon tissue, rather than pathologic degradation. Remodeling processes known to be active in young animals are for instance the changes from a high proportion of type III collagen to type I collagen and from a unimodal fibril diameter distribution to a bimodal one.[11]

The concentration of HP crosslinks increased during the first year and remained stable thereafter. This finding is in line with earlier observations in articular cartilage,[4] where changes in HP crosslink concentrations continue for a longer time than for most other collagen biochemical variables.

Pentosidine crosslinks are products of the (time-related) process of nonenzymatic glycation and have been used to assess the remodeling rate of the collagen network.[31] A linear time-related increase in pentosidine concentration in tissue is indicative of a low metabolic rate. Pentosidine concentrations were stable in the sesamoid region during the first year of life and started to increase afterwards. In the metacarpal region, pentosidine concentrations increased earlier and reached a plateau at 5 months of age, which was the same concentration of pentosidine as in the sesamoid region at 36 months of age. These differences may be related to the dissimilar influence of birth and the relatively sharp increase in weight during the first year on the metacarpal and sesamoid regions. The sudden change to weight bearing may have influenced the sesamoid region, which until birth did not experience compressive forces, more than the metacarpal region, in which tensional forces were present from the early embryonic stage. This may have induced higher metabolic activity and hence less accumulation of pentosidine directly after birth in the sesamoid region, compared with

the metacarpal region, explaining the initial plateau in the curve.

For GAG and collagen concentrations, another pattern was found. Here, significant differences were found between the 2 investigated sites at birth that either resolved or became reversed during the juvenile period, but returned in mature horses. These changing patterns may have to do with the different biomechanical environments in utero, during the phase of rapid growth, and in mature horses.

Concentrations of GAG are known to be associated with the determination of collagen fibril diameter.[32] The immediate increase in tensile forces that is most evident in the metacarpal region is known to result in a strong increase in tendon fibril diameter during tendon matrix maturation,[11,33,34] which may thus be related to the rapid increase in GAG concentrations in the first 5 months in the metacarpal region.

In collagen, the decrease in concentration in the metacarpal region after birth may represent the rapid restructuring of the collagen fibrils, especially in this portion of the tendon. The gradual increase after 5 months of age may reflect the functional response to principally tensional demand. In the sesamoid region, the gradual decrease of collagen concentrations after 12 months of age probably reflects a certain downregulation for collagen in favor of the production of fibrocartilage components at sites that face compression.[35]

The DNA concentrations decreased after birth in both the metacarpal and sesamoid regions. This may be related to the overall decrease in metabolic activity and to the increase in importance of the ECM, as functional demand increases with age. It is not clear why cellularity is high in the sesamoid region in neonates. The high cellularity in the metacarpal region in mature horses is likely related to the frequent occurrence of microdamage in this region.[36]

Hyaluronic acid concentrations seem to react quite rapidly to loading. The substantial increase in the sesamoid region in the early juvenile period and the smaller increase in the metacarpal region may reflect cell migration and proliferation for the accomplishment of structural changes[37] and thus differences in metabolic status of those regions. Not only GAG concentrations but also HA concentrations are associated with the determination of collagen fibril diameter.[32] While the primary stage of fibril growth is occurring, the transverse or circumferential growth of collagen fibrils is limited by the HA-rich matrix so that only small-diameter fibrils are formed.[32] The decrease of HA concentrations until 3 years of age, which is the age most horses are taken into training, might be related to the lateral growth of fibrils, which is a response of the cells to a changing mechanical or microelectrical environment.[38]

It is concluded that, in contrast to the situation in cartilage, the tendon does not have a homogeneous biochemical composition at birth. The idea of a blank tendon, which is most probably caused by movements in utero, therefore does not apply, but the more general concept of functional adaptation[3,39] seems to hold. After birth, the foal passes through various phases characterized by different biomechanical challenges that seem to influence tissue composition. The transitions between these phases are either sudden or gradual, which probably influences the speed of adaptation. Adaptation can go quickly, especially in young foals. It has been shown that within 1 week after birth, the energy-storing flexor tendon increased substantially in size in the neonate, with concomitant increased strength and stiffness.[a] Knowledge of these processes, which take place before a steady state is reached in mature horses, is crucial. Understanding of the processes that determine the ultimate quality of one of the most vulnerable structures in horses may help in the development of optimal exercise regimens to prepare young horses in the best possible way for future athletic activity.

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