Histoarchitecture of the fibrillary matrix of human fetal posterior tibial tendons

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Adult tendons are highly differentiated. In mature individuals, tendon healing after an injury occurs through fibrotic tissue formation. Understanding the intrinsic reparative properties of fetal tendons would help to understand the maturation tissue process and tendon tissue repair. The present study evaluated the evolution of histoarchitecture, cellularity and the distribution of collagens I, III and V in the posterior tibial tendon in human fetuses at different gestational ages. Morphological profiles were assessed in nine fresh spontaneously aborted fetuses (Group I: five fetuses aged between 22 and 28 weeks of gestation; Group II: four fetuses aged between 32 and 38 weeks of gestation), characterized by a combination of histology, fluorescence and immunohistochemistry. In Group I, the posterior tibial tendon showed statistically significant greater cellularity and presence of collagen III and V than in Group II tendon, which showed a predominance of collagenous I and a better organization of the extracellular matrix compared with Group I tendons. In addition, a statistically significant higher rate of CD90, a marker of mesenchymal cells, was found in Group I tendons. In fetuses with gestational age between 22 and 28 weeks, the posterior tibialis tendons showed a thin and disorganized fibrillar structure, with an increase in collagen III and V fibers and mesenchymal cells. In the posterior tibialis tendons of fetuses with gestational age between 32 and 38 weeks, the fibrillar structure was thicker with a statistically significant increase in type I collagen and decreased cellularity.

The posterior tibial tendon (PTT) is the main inverter of the ankle and foot and the primary dynamic stabilizer of the medial plantar arch, acting in conjunction with the capsule-ligamentous passive stabilizers1. Posterior tibial tendinopathy is very common, especially in women, in part due to hormonal changes related to the post-menopausal period, with a prevalence of 10% in the seventh decade of life, restricting activity and affecting the quality of life of this population1–4. New factors have been implicated in the genesis of pathology, with accelerated development of new techniques, implants, and rehabilitation protocols; however, the posterior tibial tendon remains the primary dynamic arch stabilizer, and tissue healing remains a fundamental factor to achieve optimal clinical results5.

Adult tendons are highly differentiated structures that, after an injury, react through a reparative process that results in the formation of a fibrotic scar due to its low regeneration capacity6,7. Tissue alterations are represented by degenerative tendinosis characterized by: neovascularization, mucin deposition and an increase in the quantity and activity of fibroblasts, reflected respectively by the increase in cellularity, proline and hydroxyproline in the

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were immersed in buffered formalin 10% with a Sodium Phosphate buffer solution at pH = 7.0 for staining with insertion (Fig. 1). For the purposes of the histological and morphometric analyses, human fetal tendon samples were harvested from the teromedial region of the ankle. The posterior tibial tendon was identified and was harvested from its origin to the aid of a 15 mm curved scalpel blade, we made an incision of approximately 1 cm over the TTP in the posterior tibial tendon in human fetuses of different gestational ages.

During fetal development, tendons undergo significant changes that transform a plastic tissue into a highly differentiated structure. Tendon maturation is characterized in some studies by its morphological and histological characteristics, which include cellularity and the organization and distribution of collagens, as well as evaluating the gene expression of factors that correlate with fetal maturity such as Scleraxis B and Tenomodulin. In general, during development, these genes suffer a reduction in expression, which would justify the lower tissue regenerative capacity after maturation. However, most studies on tendon organization and function analyze the tissue of mice, rats, horses, and sheep, with a limited number of studies on human fetal tendon histomorphology.

We hypothesized that there is a difference in the composition and arrangement of the extracellular matrix of the posterior tibial tendon of human fetuses at earlier stages when compared to the lateral gestational ages. The present study evaluated the evolution of histoarchitecture, cellularity, mesenchymal stem cell markers, and the distribution of collagens I, III and V in the posterior tibial tendon in human fetuses of different gestational ages.

**Methods**

Our institution's scientific and research ethics committees approved all the procedures described in the present study (Scientific Committee of the Institute of Orthopedics and Traumatology, Faculty of Medicine, University of São Paulo under protocol number 1340 and a consubstantial opinion of the Ethics and Research Committee under number 3.031.872). Signed parental informed consent was obtained to examine fetuses obtained from spontaneous abortions and stillbirths. All experiments were performed in accordance with relevant guidelines and regulations, including the Declaration of Helsinki. Gestational ages were determined by the head, buttock length, foot length and fetal weight, and the gestational age mentioned by the parents when signing the consent form (ICF).

**Inclusion criteria.**Spontaneously aborted fetuses with a gestational age between 22 and 38 weeks.

**Non-inclusion criteria.** Fetuses less than 22 weeks old, given the difficulties in the dissection of the early specimens and the higher chance of malformations or anomalies evident on external inspection or after autopsy. We obtained a total of 9 fetuses divided into two groups, Group 1: 5 fetuses—22–28 weeks of gestation (mean 24.80 weeks gestational age) and Group 2: 4 fetuses—32–38 weeks of gestation (mean 33.75 weeks of gestational age).

The PTT was collected from both sides of each fetus. These specimens underwent histoarchitecture and histomorphometric analysis of the extracellular matrix components, including collagens I, III and V.

**Dissection and specimen preparation.**We performed the dissections and resections of the tendons of human fetuses’ fragments at the Death Verification Service of the Capital of the University of São Paulo. With the aid of a 15 mm curved scalpel blade, we made an incision of approximately 1 cm over the TTP in the posteromedial region of the ankle. The purposes of the histological and morphometric analyses, human fetal tendon samples were immersed in buffered formalin 10% with a Sodium Phosphate buffer solution at pH = 7.0 for staining with hematoxylin–eosin (H&E) and Picrosirius. The solution consists of Sodium Chloride (Code S9888), Potassium Chloride (Code P3911), Potassium Phosphate Monobasic (Code P5655), and Dibasic Sodium Phosphate (Code P71645).

**Optical microscopy.** Tendon sections (3–4 µm) were used in the deparaffinization process in ethanol and hydrated in graded ethanol. Subsequently, they were stained with hematoxylin guidance (H&E) and instrument evaluations and evaluations at Bx51, Tokyo, Japan) for B51 structure and tissue cell age. Subsequently, from an analysis of tissue collagen in optical analysis, samples were stained by Picrosirius, which is a selective connective tissue staining that allows a qualitative analysis of the collagen fibers of connective tissue, prepared from Sirius red 0.2% here epicry acid solution (Direct Red 80, CI375, Milwaukee, WI).

**Collagen types immunofluorescence.** For immunostaining of collagen types I, III and V, 3–4 µm sections of tendon tissue samples were adhered to slides with aminosilane (Sigma Chemical Co.; St. Louis, Missouri, USA). They were immersed in xylene and rehydrated in decreasing concentrations of ethanol. The immunogenic sites were exposed to enzymatic treatment with porcine gastric mucosa pepsin (P7000; Sigma Chemical Co.; St. Louis, Missouri, USA) at 10 mg/ml concentration in 0.5 N acetic acid, pH 2.2 for 45 min, at 37 °C. After successive washes with PBS, the slides were incubated in 5% bovine albumin (BSA) and diluted in phosphate buffer pH 7.0 for 30 min. They were subsequently incubated overnight at 4 °C with polyclonal rabbit anti-human Collagen I (1:100, Rockland, Carlsbad, CA, USA), anti-human Collagen III (1:200, Rockland, Carlsbad, CA, USA), and

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anti-human Collagen V antibodies (1:2000, Rockland, Carlsbad, CA, USA) diluted in BSA (bovine albumin, Sigma Chemical Co., St. Louis, MO, USA) and stained with goat antibody ALEXA FLUOR 488 anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted 1:200 in a PBS solution containing 0.006% Evans blue. As a negative control, the primary antibody was replaced by PBS. After washing with PBS/0.05% Tween20, the slides were again incubated with 1 µg/ml Hoechst 33258 bisbenzimide (DAPI) (Invitrogen, Carlsbad, CA, USA) to evidence the cell nucleus. Finally, slides were mounted with a glycine buffer in PBS (v/v) and analyzed under a fluorescence microscope (Olympus BX51, Olympus Co, St Laurent, Quebec, Canada)12,25.

Mesenchymal stem cell immunostaining. To analyze mesenchymal stem cells from the tendon tissue, 3–4 µm sections were adhered to slides with aminosilane (Sigma Chemical Co.; St. Louis, Missouri, USA). The slides were immersed in xylene and rehydrated in decreasing concentrations of ethanol. Subsequently, a 0.3% hydrogen peroxide solution was used four times for 5 min to inhibit the activity of endogenous peroxidase, and the antigenic recovery was immediately processed. The immunogenic sites were exposed to enzymatic treatment with porcine gastric mucosa pepsin (P7000; Sigma Chemical Co.; St. Louis, Missouri, USA) at a concentration of 0.4% in glycine buffer pH 2.2 for 30 min at 37 °C, and then incubated with CD90 monoclonal primary antibody (Abcam Ab 92574) diluted 1:200 in 0.01% BSA, overnight at 4 °C. According to the manufacturer’s recommendations, the reaction was developed using a biotin–streptavidin–peroxidase (Novolink Polymer Detection Systems Kit, Leica Biosystems, UK). After that, 3,3 dianinobenzidine (Sigma Chemical, St Louis, MO) was used as the chromogen and counterstained with Harris hematoxylin (Merck, Darmstadt, HE Germany). The IgG isotype was used as a negative control. To access uniform and proportional tendon samples, ten fields were randomly analyzed in the tendon at ×1000 magnification for CD90 expression, and the cells count was performed by a manual point count in each field system software Image Pro-Plus 6.0), composed of an Olympus camera (Olympus Co, St Laurent, Quebec, Canada) coupled to an Olympus microscope (Olympus BX51), from which the images were sent to an LG monitor utilizing a digitizing system (Oculus TCX, Coreco, Inc., St. Laurent, Quebec, Canada) The results are reported as the percentage of positive cells in the tendon per micrometer square26.
Histomorphometry of tendons. Photomicrographs at magnification 400× were obtained from ten non-overlapping fields of view per section under a fluorescence microscope (Olympus BX51, Olympus Co, St Laurent, Quebec, Canada). The images were processed through Image Pro-Plus 6.0 software (NCH Software Inc., Greenwood Village CO, USA), and the immunofluorescence density of collagen I, III, and V fibers were measured. The threshold for identification of collagen fibers was given for all slides after the contrast was increased to the point where the fibers were clearly identified as green bands. Its density was expressed as the ratio between the measurement of the fibers by the total area studied 100×. The microscopic fields of the slides were quantified, and the results were shown as the average of these fields.

Statistical analysis. Data are presented as the mean ± standard deviation of the mean. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Prism, Inc., San Diego, CA, USA), and P-values < 0.05 were considered significant. Statistical differences between groups were determined by the Mann–Whitney U test for an area of the fraction occupied by mesenchymal cells and immunofluorescence for collagen I, III and V of the tendons. Post-hoc tests, corrected for multiple comparisons with the Tukey-Kramer fit, were used to locate significant differences when significant main effects or interaction effects were shown. Spearman correlation was performed between cells and collagen types.

Results
Cell distribution in human fetal tendons. Group I (22–28 weeks) had tendons with a large number of cells with an asymmetric distribution, mainly in the endotenon region, around the tendinous tissue and immersed in collagen bundles, following a pattern of thick scaffolding. In Group II (32–38 weeks), tendons with a lower degree of cellularity were found, with increased parallel or linear orientation of collagen bundles (Fig. 2A). Regarding the presence of mesenchymal cells, we found an intense expression of CD90 in human fetal tendon samples from Group I (22–28 weeks; n = 5) and Group II (32–38 weeks; n = 4) show typical histoarchitecture appearance in H&E preparations. At lower magnification, a large number of cells immersed in the fibrillar matrix can be seen in Group I in relation to the tendons in Group II. At higher magnification, note the cells distributed among the collagen fibers in the tendons of Group I (arrows) compared to the tendons of Group II (arrows) (insert) arranged in a linear orientation along the collagen bundles. (B) Immunostaining of CD90 in Group I compared to human fetal tendons in Group II. Note a high expression of CD90 positive cells throughout the tendon matrix in human fetal tendons in Group I (arrows) (insert) and in (C) Graphic representation of CD90 measurement (mean ± standard deviation), showing a significant increase in the number of positive cells in Group I compared to Group II (7.20 ± 1.06 vs. 4.01 ± 0.51 cell/μm²; p < 0.015). This graph shows the mean and the value found in the analysis of the dispersion of each specimen in the sample. GraphPad Prism 6.0 software; Mann Whitney U test; P-values < 0.05 were considered significant.
tendons in Group I compared to immunostaining in Group II. A high expression of CD90-positive cells in the tendon tissue is characterized by intense cytoplasmic immunostaining in these cells (Fig. 2B). Furthermore, the histomorphometric results showed more CD90 in human fetal tendons in Group I compared to Group II (7.20 ± 1.06 vs. 4.01 ± 0.51; P < 0.0159), this marker was found mainly in the endotenon (Fig. 2C).

**Collagen molecular architecture and cells amount depends on gestational fetus age.** In Group I, a network of reddish-orange birefringent fibers in Picrosirius staining was identified, with a fragmented and broken pattern covering the entire surface of the tendon (Fig. 3A,B). In contrast, the collagen fibers in Group II human fetal tendons showed a parallel or linear orientation of the tissue collagen bundle (Fig. 3CD).

Human fetal tendons in Group I showed greatercellularity, with a disordered pattern in all tendon tissues (Fig. 3E,L,M). In Group II tendons, the number of cells decreased, and a linear and uniform pattern oriented in parallel, following the tissue-oriented fibrillar matrix, was evident (Fig. 3G,K,O).

Through histomorphometric analysis, there was a significant increase in cell content in human fetal tendons in Group I compared to tendons in Group II (23.35 ± 1.74 vs. 16.07 ± 0.77; p < 0.0357) (Fig. 3Q). The immunofluorescence analysis of collagen I showed green fluorescence for collagen type I, with diffuse fiber distribution in all areas of the tendon for Group I. On the other hand, tendons in Group II exhibited marked green fluorescence for collagen type I, with parallel or linear orientation of the collagen bundles (Fig. 3F,H). Quantitative results showed a significant increase in collagen I in Group II tendons compared to Group I tendons, respectively (51.24 ± 1.71 vs. 31.11 ± 1.74; p < 0.0159) (Fig. 3R). In addition, immunofluorescence analysis of collagen types III and V showed a finely reticulated network of collagen fibers in human fetal tendons in Group I, with diffuse fiber distribution in all tissue areas (Fig. 3J,N). In contrast, Group II tendon specimens showed prominent fluorescence for collagen types III and V arranged in a fine and regular fibrillar fashion with better organization of the extracellular matrix (Fig. 3L,P). There was a significantly greater amount of type III collagen in the tendons of Group I compared to group II, respectively (31.19 ± 2.10 vs. 17.02 ± 1.37; p < 0.015), as well as type V collagen (12.52 ± 0.76 vs. 4.39 ± 0.76; p < 0.016) (Fig. 3S,T).

**Discussion**

As in other connective tissues, the human posterior tibial tendon undergoes morphological and molecular changes during its development, which involve both its cells and the extracellular matrix. The process of in utero maturation of the posterior tibial tendon is accompanied by a progressive decrease in cellularity and modification of tissue structure. We observed a significantly greater number of tenocytes per field in the immature tendon, confirming the findings of studies in equine and sheep tendons. This is the first study to perform this evaluation in the posterior tibial tendon of human fetuses.

During the process of fetal development, there was a reduction in the cellularity of the posterior tibial tendon that accompanied tendon maturation. The endotenon was more robust in younger fetal tendons compared to more mature ones, and cell characteristics also changed as the tendon matured. In fact, the specimens from Group I presented heterogeneous cell shapes and in greater quantity when compared to those from Group II, which presented cells with a more fusiform shape and in smaller numbers. Avilion et al. and Russo et al. have described similar findings in animal tendon specimens. The fact that there is no evidence of pyknosis or nuclear fragmentation under microscopy makes us propose that this reduction is unrelated to apoptosis.

Hosaka et al. and Chuen et al. present similar results in horse digital flexor and human patellar tendons, respectively. In our study, the posterior tibial tendon of fetuses at different gestational ages presented collagen types I, III and V in different amounts and a distinct distribution pattern. Tresoldi et al. state that fibrillogenesis begins during embryogenesis and continues after birth with the assembly of type I collagen molecules, which follow linear and lateral growth associated with collagen interactions with proteins such as other collagens and proteoglycans. Initially, collagen molecules assemble to form immature fibril intermediates, and after this molecular assembly, fibril intermediates assemble end-to-end to form longer fibrils, consistent with mechanically functional mature fibrils. These data corroborate findings in the posterior tibial tendon of human fetuses, in which the amount and pattern of organization of type I collagen increased in more mature fetuses compared to younger ones.

Younger fetuses had collagen type III in greater amounts compared to more mature fetuses. Tozer and Duprez state that collagen III expression gradually decreases during development and that its high expression in the early stages suggests a role in the initial assembly of the fibril. Furthermore, its expression is elevated after tissue injury, suggesting that this collagen may play a role in the healing process, perhaps by stimulating fibrillogenesis.

Romican et al. evaluated that collagen III, in the form of procollagen III, can regulate the diameter of collagen I fibrils, coating their surface, thus allowing longitudinal growth, but not lateral growth of fibrils with eventual thickening of the tissue tendon.

Hansen et al. demonstrate that type V collagen plays the role of a molecular ligand between collagen I fibrils or between fibrils and macromolecules depending on their respective distribution in different tissues. Thus, it is associated with the quantity and quality of the distribution of collagen I fibrils and, consequently, of the tendon. Connizzo et al. demonstrated that, after the deletion of the genes responsible for the production of type V collagen in mice, an assembly of fibrils of large diameter and wide distribution occurs, characteristics similar to the fibrils produced in connective tissues with low concentrations of this collagen. This suggests that type V collagen levels regulate fibril diameter and that its reduction may be sufficient to alter fibril assembly so that abnormally large diameter fibrils are deposited in the matrix. In our study, younger fetuses had a higher amount of type V collagen when compared to more mature fetuses. This corroborates the data presented in the literature and suggests a greater ability to regulate and organize collagen fibrils in younger tendon tissue samples showed a greater amount of cells immunostained for CD90, one of the markers of mesenchymal stem cells, compared to more mature tendons. The presence of these cells was
observed mainly close to the blood vessels, as demonstrated by Lui et al. in their evaluation of adult tendons, suggesting that they may respond to local and systemic regulatory signals. Russo et al. also demonstrated a high expression of markers that characterize mesenchymal cells in fetal tendons of younger sheep when compared to more mature and adult fetal tendons. Adult mesenchymal stem cells are able to differentiate into: bone, cartilage, muscle, medullary stroma, tendon, ligament, fat, and other connective tissues in a sequence of lineage transitions. Caplan et al. demonstrate that bioactive molecules secreted by these cells were able to promote neovascularization, migration, immunoregulation, cell proliferation, synthesis, and remodeling of the extracellular matrix in the tissue. However, he states that it is still unclear whether tendon stem cells work to replace damaged tendons or to establish a microenvironment for injury repair. Still, both can occur in vivo after tendon injury.

Figure 3. Collagen molecular architecture and cells distribution. (A,B) Note reddish-orange birefringent collagen fibers analyzed by picrosirius under polarization in Group I (n = 5) human fetal tendons with a fragmented and disarranged pattern. In contrast, the collagen fibers stay in parallel or linear orientation in Group II (n = 4) tendons, (C,D) (arrows). (E,I,M) fluorescence microscopy by DAPI staining to identify the cell nucleus, with a large amount of cells with diffuse pattern in Group I tendons and in (G,K,O) demonstrate Group II tendon specimens with uniform and linear arrangement of the nuclei (arrows). (F,J,N) Immunostaining to collagen I, III and V visualized under fluorescence microscopy of Group I, demonstrating the reticulated pattern of collagen types. In contrast, (H,L,P) uniform green fluorescence and parallel orientation of collagen type I, III and V fibers in all areas of Group II tendons. (Q) A graphic representation of the total number of cells in Group I compared to Group II tendons (23.35 ± 1.74 vs. 16.07 ± 0.77; p < 0.04). A graphic representation of the amount (percentage of total tendon area, mean and standard deviation) of collagen I (51.40 ± 1.71 vs. 31.11 ± 1.74; p < 0.016) (R), collagen III (31.19 ± 2.10 vs. 17.02 ± 1.37; p < 0.015) (S) and collagen V (12.52 ± 0.76 vs. 4.39 ± 0.76; p < 0.016) (T) in the Group I in compared to Group II tendons. GraphPad Prism 6.0 software: Mann Whitney U test; P-values < 0.05 were considered significant.
In the present study, the characterization of mesenchymal stem cells aimed to identify whether the constitution of the fibrillar matrix of the tendon could be linked to the increase of these cells during the development of the tendon tissue of the human fetus. However, other markers that characterize this cell lineage would be necessary to support this hypothesis. On the other hand, the indication of a greater amount of cells that express CD90 may be a strong indication of the participation of these cells in the maturation of the tendon of the human fetus.

Goldman et al. and Russo et al. have already demonstrated a greater association of the amount of TGFβ with the production of collagen I and a greater association with mesenchymal stem cells in the endotenon. Our attempt at immunostaining TGFβ in a human tendon by immunohistochemistry showed a nonspecific and difficult to interpret the result in tissue. We believe that the fact that the fetuses were removed from the death verification service may have had some influence on this issue.

A deeper understanding of the intrinsic properties of the response of fetal tissue to injury may allow for the modulation of the response of mature tendon tissue to injury. There are undoubtedly many more molecules involved in this response than can be examined in a single investigation, which is a weakness of the present work. Nevertheless, our study begins to provide information on the complex mechanisms that control the process of maturation and organization of human tendons, and the information from this project can be the starting point for the development of innovative therapies to minimize the formation of scars after tendon injury in humans.

Data availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions
All authors conceived the studies, and R.S.M., V.L.C. and D.L.R. collected the data. R.S.M., W.R.T., and D.L.R. analyzed the results. R.S.M., W.R.T., and D.L.R. wrote the manuscript, and all authors reviewed and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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