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Amniotic Membrane and Stem Cells Improve the Immunohistochemical Profile of Injured Rat Achilles Tendons

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Abstract

Tendon disorders are common and have a major socio-economic impact. Current treatments (drugs, physiotherapy, surgery) do not provide lasting relief, leading to chronicity and recurrence. In this context, studies on regenerative therapies, such as stem cells, platelet-rich plasma and natural and synthetic membranes, have shown promising results in the treatment of tendon lesions. The present study analyzes the tissue response to a combination of bone marrow mononuclear cells BMMCs) and human decellularized amniotic membrane for treatment of Achilles tendon lesions in rats. Forty male Wistar rats were randomized into four treatment groups: SC (stem cells), AM (amniotic membrane), SC + AM (stem cells + amniotic membrane) and C (control). All underwent Achilles tendon sectioning and tenorrhaphy. In the AM and SC + AM groups, amniotic membrane was sutured over the lesion after the tendon was sutured; in the SC and SC + AM groups, 2 ml of autologous blood from the iliac crest containing BMMCs was applied around the lesion. Group C animals received only 2 ml of 0.9% saline around the lesion. After four weeks, the animals were euthanized, and the tendons were sent for histological analysis (*Picrosirius Red*) and immunohistochemistry (IL-6, IL-4 and IL-13). Analysis of type I and type III collagen fibers showed no differences between groups. However, the SC + AM group showed a better immunohistochemical profile, with greater expression of IL-4 and IL-13. In this experiment, animals treated with amniotic membrane and autologous stem cells had a better immunohistochemical profile than controls, with increased expression of cytokines associated with tissue repair and organization.

Keywords: achilles tendonitis; tendonitis treatment; cell therapies; Synthetic membranes

1. Introduction

Tendon disorders are common in clinical practice and have low healing potential in both acute and chronic injuries [1,2]. Current treatments, based on anti-inflammatory drugs, rehabilitation programs, and surgery, fail to provide lasting and significant relief, and there is no therapeutic consensus or modality considered *gold standard* [3,4].

Within this context, advanced therapies have emerged as promising options [5]. Among cell therapies, bone marrow mesenchymal stem cells (BMMCs) are a type of stem cell (SC) that can differentiate into tenocytes and induce the formation of linearly arranged type I collagen, as well as increase resistance to stress modulus, tension, and deformation, improving the biomechanical characteristics of tendons [6–8].

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Synthetic membranes, such as silk, or natural membranes, such as amnion, have the primary function of providing a structural framework. Amnion, a connective membrane associated with the placenta, has anti-inflammatory and antimicrobial properties and low antigenicity when decellularised [9]. In controlled studies, amniotic membrane fragments placed in injured tendons were associated with better organization of type I collagen compared to the control group [10–12].

Finally, the use of target therapies to block pro-inflammatory interleukins and their receptors is already a reality in the treatment of several conditions, including cancers (such as some types of melanoma) and autoimmune diseases (such as rheumatoid arthritis) [13,14].

Regarding the molecular process of tissue repair [15], Interleukin-6 (IL-6) is an acute-phase pro-inflammatory cytokine, but it can persist late in inflammatory processes, assuming an immunomodulatory function and being related to tendon healing. [16]. In canine and murine models, its gene expression is increased in animals subjected to tendon injury, similarly to how, in humans, pertitendinous)infiltration of IL-6 stimulates collagen synthesis [17].

Interleukin-4 (IL-4) is also involved in collagen production by fibroblasts. It forms negative feedback with IL-6 and interferon-gamma, antagonizing their initial pro-inflammatory action, and appears in later inflammatory processes, controlling the initial inflammatory process for effective tissue repair [18]. IL-4 does not act directly in the collagen formation process, but presents positive feedback with Interleukin-13 (IL-13) [19].

IL-13, closely related to IL-4, regulates the breakdown of type III collagen (disorganized, initial) and the consequent deposition of type I collagen (mature, linear) through the subsequent activation of extracellular matrix metalloproteinases (MMPs). One of the main ways in which IL-13 promotes collagen synthesis is through the activation and amplification of TGF-β (tumor growth factor beta) signaling, which stimulates fibroblasts. MMP-9, mediated by IL-13, cleaves latent TGF-β into active TGF-β and stimulates its expression. However, fibroblasts also respond to the direct action of IL-13 and IL-4, independently of the action of TGF-β. [20].

Since mesenchymal stem cells are capable of remodeling injured tendons, increasing biomechanical properties (maximum stress, modulus, and strain), tenocyte number, and number and quality of mature collagen fibers [5–8], while amnion-derived membranes and cells have been reported to have multipotent differentiation capacity [9–12], both show complementary therapeutic effects, justifying the study of their combined use in the treatment of inflammatory tendinopathies, for which current therapies have a high failure rate [3,4].

Therefore, this study aimed to assess whether a combination of BMMCs and AM induces a synergistic response in the treatment of Achilles tendon rupture in rats.

2. Materials and Methods

Following approval by the Ethics Committee of the Victor Ferreira do Amaral Maternity Hospital (registration 01238), placentas were collected from two parturient women. The animal experiment was approved by the Animal Care and Use Committee of the Pontifical Catholic University of Paraná (CEUA-Pr 01637), and followed international animal welfare standards (*ARRIVE* guidelines). Forty male Wistar rats, weighing an average of 350 g, were used.

EXPERIMENTAL LESION MODEL

The rats were anesthetized intraperitoneally with 5% ketamine hydrochloride (Vetanarcol™, Konig do Brasil Ltda., Brazil) at a dose of 80mg/kg combined with 2% xylazine hydrochloride (Rompun™, Bayer S.A, Brazil) at a dose of 10mg/kg. Once anesthesia had been achieved, as verified by pupillary reflex and muscle relaxation, the surgical site was shaved and tenotomy was performed, as demonstrated in Figure 1A.

In brief, a linear skin incision was made in the right hind leg of each rat, the Achilles tendon was individualized, bisected completely at the middle third, and sutured with 5-0 polypropylene (PolipropypointPoint Suture, Brazil).

After surgery, animals were randomized into four treatment groups (n=10 each):

- SC group, (BMMCs only;

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- AM group, AM only;
- SC + AM group, BMMCs + AM;
- C group (control), 0.9% saline solution.

For animals in the AM and SC + AM groups, after after the Achilles lesion had been bridged with polypropylene 5-0 sutures, a decellularized amniotic membrane was was fitted and sutured to the tendon along its edges with4 stitches at its ends (also with 5-0 polypropylene) The technique for obtaining, preparing, and storing the amniotic membrane is described below.

Skin closure was performed with 5-0 polypropylene, after which animals in the SC and SC+AM groups were percutaneously infiltrated with stem cells in the Achilles peritendon (Figure 1B), while animals in the C group were percutaneously infiltrated with 2 mL of 0.9% saline solution (the technique for stem cell isolation and preparation is also described below).

Four weeks after induction of injury, the animals were euthanized according to the anesthesia protocol described above, and the tendons were resected and sent for collagen and immunohistochemical evaluation (Figure 1C and 1 D).

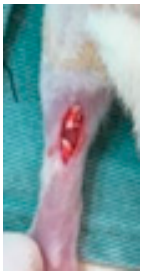

Fig 1 A

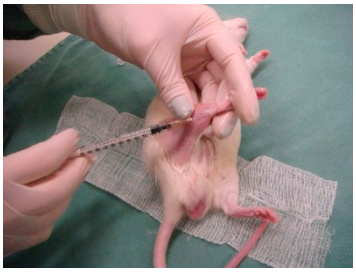

Fig 1B

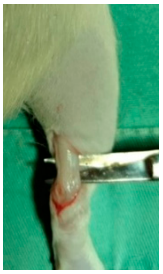

Fig 1C

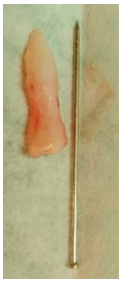

Fig 1D

Figure 1. Experimental steps of the animal model lesion. Fig 1A: Achilles tendon following skin incision and tenotomy. Fig 1B: Percutaneous application of stem cells/ 0.9% saline solution. Fig 1C: Intraoperative image of the tendon at 4 weeks, at the time of euthanasia and tendon resection. Fig 1D: Resected tendon specimen.

ISOLATION AND PREPARATION OF BONE MARROW MONONUCLEAR CELLS

For rats in the SC and SC + AM groups, the procedure began with the collection of blood from their iliac crests for preparation and isolation of stem cells according to the technique described by Boyum, namely separation by density gradient using Iscove's Modified Dulbecco's Media (IMDM) and density gradient separation solution (Ficoll-Hypaque - Ficoll® Paque Plus GE17-1440-02, liquid, sterile, endotoxins < 0.12 EU/mL, density: 1.077 g/mL, pack of 6 × 100 mL) [21], as explained below.

To obtain the BMMCs, the right iliac crest of each animal was punctured with a 14-gauge needle and approximately 2-3 ml of blood aspirated into anticoagulant-containing tubes, isolated by Ficoll density gradient, and cultured in IMDM medium supplemented with antibiotics (penicillin and streptomycin 1%). After counting in a Neubauer chamber, the cells were resuspended in sterile PBS ($1x10^5$ cells/µL) and injected in the site of the tendinous lesion with a syringe and insulin needle.

AMNIOTIC MEMBRANE PREPARATION

Fresh amniotic membrane (AM) was obtained after caesarean deliveries from human maternal donors with negative serologies for HIV, hepatitis B, hepatitis C and syphilis.

After delivery of the placenta, blood clots were immediately removed by washing the placenta with phosphate-buffered saline solution (PBS) pH 7. 2, containing 100u/ml penicillin and 100mg/ml streptomycin (Gibco®).

Decellularization of the AM was performed by removing amniotic epithelial cells from the membrane using 0.01% sodiumdodecyl sulphate and 0.01% sodium deoxycholate in PBS and incubated at a rotation speed of 100 rpm at 37º C for 24 h in a class II BioSAFE biological safety cabinet (Veco®). The AMs were then washed three times with PBS for further decellularization and preserved in PBS at 4° C [22,23], and Figure 2 shows the final appearance of the decellularized AM.

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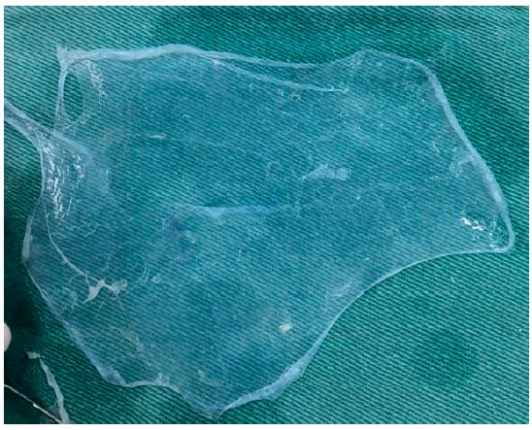

Figure 2. Final aspect of the amniotic membrane, after preparation and decellularization.

HISTOLOGICAL AND IMUNOHISTOCHEMICAL ANALYSISHistological sections were mounted for *Picrosirius Red* staining (Direct Red: Aldrich Chemical Company Inc., Milwaukee, WI, USA) to specifically characterize collagen fibers type I (red) and type III (green), under a circular polarization lens. 10 high-power fields (HPF) generation was performed.

The immunohistochemistry technique was used to identify the immunoexpression of interleukin-4 (IL-4, PAS-25165, 1:200, Thermo Fisher Scientific AB_2542665), interleukin-6 (IL-6, MP5-2OF3, Thermo Fisher Scientific, AB_469216) and interleukin-33 (IL-33, P130-E, 1:600, Thermo Fischer Scientific, AB_223471ABclonal, Manhattan Beach, CA). Immunohistochemistry was carried out as follows. Primary antibodies were incubated in a humid chamber at 2–8°C overnight. Subsequently, the secondary polymer (Reveal Polyvalent HRP-DAB Detection System, Spring Bioscience, Pleasanton, CA) was applied to the sections for 25 minutes at room temperature. Visualization was achieved by exposure to the 2,3-diaminobenzidine (DAB) complex with hydrogen peroxide substrate for sufficient time to allow development of the brown chromogenic signal, followed by counterstaining with Harris hematoxylin. Specificity of the reaction was verified using a positive control tissue sample with known antibody immunoreactivity, processed in parallel with the test samples.

The slides were immunolabeled with anti-IL-4, anti-IL-6, and anti-IL-13 antibodies, scanned in an Axio Scan.Z1 slide scanner (Zeiss, Jena, Germany), and analyzed images were generated in with ZEN Blue Edition software (Zeiss, Jena, Germany). 30 HPF generation was performed randomly by the software, with no interference from the investigator. In each HPF, areas of immunoexpression were measured using Image Pro-Plus software version 4.5 (Media Cybernetics, Rockville, MD), using a semi-automated color segmentation method, in which the area of tissue immunoexpression for each biomarker was artificially delimited and quantified.

Subsequently, its area, expressed in square micrometers (μm^2) was divided by the respective total tissue area and transformed into a percentage. Finally, the arithmetic mean values expression of each biomarker of interest in the HPFs of each sample were calculated, organized in Microsoft Excel® spreadsheets, and analyzed in IBM SPSS Statistics for Windows, Version 29.0.

STATISTICAL ANALYSIS

The results were described as means, standard deviations, medians, and ranges (minimum–maximum). For comparisons among groups, the Kruskal–Wallis non-parametric test was applied. When significant, pairwise comparisons were performed using Dunn's post-hoc procedure and Bonferroni-adjusted *p*-values. The correlation between two quantitative variables was analyzed by estimating Spearman's coefficients and assessing their significance. The normality of distribution of continuous variables was assessed using the Shapiro-Wilk test. P-values < 0.05 were deemed indicative of statistical significance. Analysis was performed using IBM® SPSS Statistics v.20.0 software (IBM, Armonk, NY, USA).

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3. Results

Of the 40 animals, three died after the tenotomy and tenorrhaphy procedure, two in the SC + AM group and one in the control group. Thus, data from 37 rats were available for final analysis: SC group n = 10, AM group n = 10, SC + AM group n = 8, and C group n = 9.

For the histological assessment of type I and III collagen fibers, *Picrosirius Red* staining was carried out, in which type I collagen stains red under illumination and type III collagen stains green.

The figures below illustrate the variation in collagen composition among the samples, ranging from predominantly type I collagen (Figure 3, left), to a moderate presence of type I collagen (Figure 3, center), and predominantly type III collagen (Figure 3, right). Since no statistically significant differences were observed in the proportions of type I and type III collagen between groups, these figures are presented for illustrative purposes only.

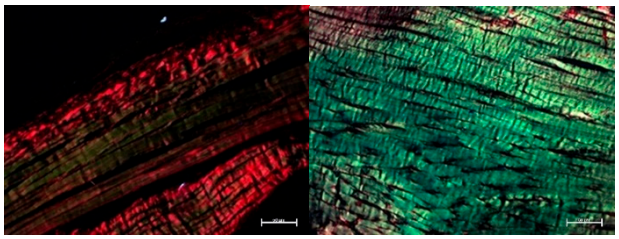

Figure 3. *Picrosirius Red* staining (400×), illustrating variation in the ratio of type I to type III collagen fibers, ranging from a low proportion of type III (green) collagen (left) to predominantly type III collagen (right).

The slides were submitted to an automated process to assess the percentage of type I collagen fibers and the percentage of type III collagen fibers, resulting in the values tabulated below (Table 1).

Table 1. Values of type I and type III collagen.

	Group	N	Mean	Standard deviation	Median	Minimum	Maximum	*p**
Col. I (%)	SC	10	84.3	18.2	90.2	37.2	97.8	
	SC + AM	8	82.0	8.4	83.1	66.3	95.3	
	AM	10	80.3	12.0	83.2	55.6	93.7	
	C	9	88.4	12.1	93.6	68.0	98.0	0.147
Col. III (%)	SC	10	15.7	18.2	9.8	2.2	62.8	
	SC + AM	8	18.0	8.4	16.9	4.7	33.7	
	AM	10	19.7	12.0	16.8	6.3	44.4	
	C	9	11.6	12.1	6.4	2.0	32.0	0.147

Table 1: Kruskall-Wallis test for statistical analysis of the percentage of type I and III collagen in the groups. $p < 0.05$ shows statistical significance.

Thus, there was no statistically significant difference in the presence of type I and III collagen between the groups.

The immunohistochemistry technique was used to identify the immunoexpression of IL-6, IL-4, and IL-13, but due to the loss of samples (slides in which the immunohistochemical staining was not fixed and therefore could not be analyzed), the final n for each group was broken down as follows in Table 2:

Table 2. Final n of each group regarding immunohistochemical analysis.

Variable	Group	N
IL-4	SC	10
	SC + AM	7

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	AM	9
	C	7
IL-6	SC	6
	SC + AM	4
	AM	8
	C	4
IL-13	SC	8
	SC + AM	6
	AM	9
	C	7

Table 2: Final n of each group for immunohistochemical staining.

Figure 4 illustrates the slide analysis, highlighting the intensity of the tissue reaction induced by the antibodies in each group.

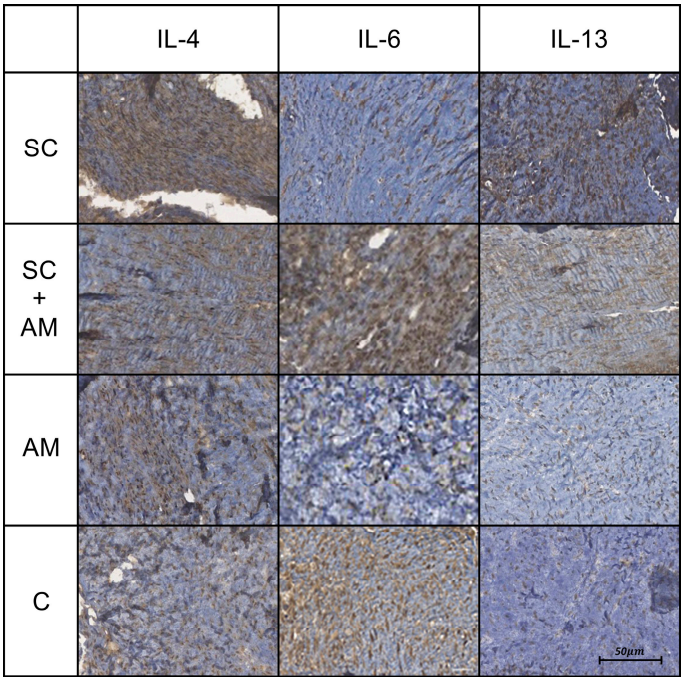

Figure 4. Images of immunohistochemical slides (x200) from each group, according to the immunohistochemistry antibodies. Scale bar in 50 µm.

Photographs were generated from each slide and read automatically, generating the percentage of area stained per total tissue area, by the immunohistochemical reaction, resulting in the values tabulated below (Table 3) and explained in the subsequent graphs (Figure 5).

Table 3. Results regarding immunohistochemical analysis.

Variable	Group	N	Mean	Standard deviation	Median	Minimum	Maximum	p*
IL-6	SC	6	0.18	0.11	0.17	0.06	0.38	
	SC + AM	4	0.93	0.83	0.77	0.24	1.94	
	AM	8	0.22	0.15	0.15	0.08	0.53	
	C	4	0.44	0.26	0.46	0.15	0.70	0.084
IL-13	SC	8	3.9	2.9	3.2	1.3	10.5	
	SC + AM	6	13.6	9.0	11.4	5.6	27.8	
	AM	9	6.8	4.2	6.0	2.0	16.4	

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	C	7	2.7	2.5	1.5	0.5	7.3	0.004
IL-4	SC	10	9,8	2.7	8.6	7.0	13.0	
	SC + AM	7	14.9	7.2	13.0	9.6	30.6	
	AM	9	9.9	5.8	9.8	3.0	23.0	
	C	7	6.6	2.5	6.0	2.9	10.5	0.011

Table 3: Results of the presence of interleukins among the groups. Kruskall-Wallis test for statistical analysis. $p < 0.05$ shows statistical significance.

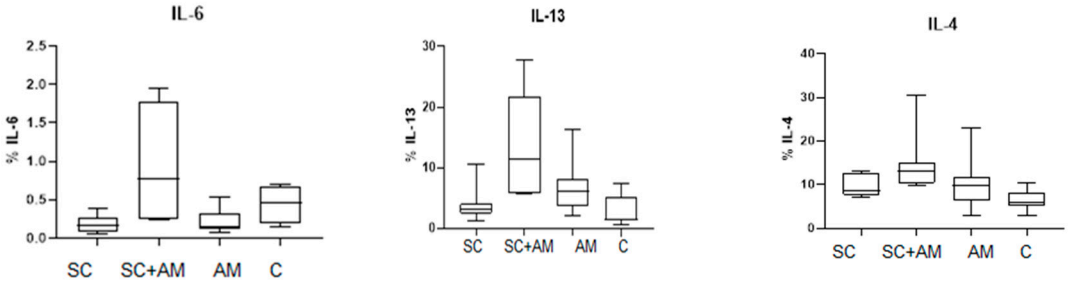

Figure 5. Left: distribution of the percentage of IL-6 among the groups. Middle: Distribution of the percentage of IL-13 among the groups. Right: Distribution of the percentage of IL-4 among the groups.

Significant differences were found between the groups for IL-13 and IL-4. Therefore, the groups were compared two by two, as shown in the table below (Table 4), which presents the p - values of these comparisons.

Table 4. Comparison of IL-4 and IL-13 between groups.

Compared groups	IL-13	IL-4
SC x C	1	0.489
SC x AM	0.733	1
SC x SC + AM	0.060	0.381
C x AM	0.101	0.985
C x SC + AM	0.005	0.005
AM x SC + AM	1	0.200

Table 4: Comparison of percentage of IL-4 and IL-13 between groups. Dunn's post hoc test. $p < 0.05$ shows statistical significance.

From the results above, the groups that showed statistically significant differences are shown in the graphs below (IL-13 and IL-4 between C and SC + AM groups, Figure 11).

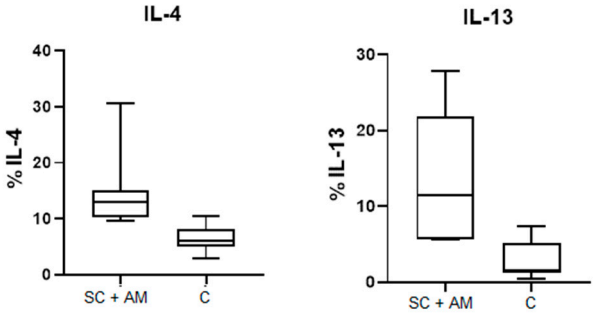

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Figure 6. Left: Statistical difference in the presence of IL-13 between the CT + MA and C groups. Right: Statistical difference in the presence of IL-4 between the CT + MA and C groups.

Finally, we carried out a statistical analysis of the correlation between variables, and table 5 below shows the estimated Spearman correlation coefficients and the *p* - values of the statistical tests for the data with significance.

Table 5. Correlation between variables with a significant difference.

Group	Variables analyzed	n	Spearman's correlation coefficient	*p* - value
MA	IL-4 x collagen I (%)	9	-0.65	0.058
	IL-4 x collagen III (%)	9	0.65	0.058
All	IL-13 x collagen I (%)	30	-0.35	0.057
	IL-13 x collagen III (%)	30	0.35	0.057

Table 5: Spearman's correlation coefficient. $p < 0.05$ shows statistical significance.

The correlation coefficient between IL-4 and IL-13 and collagen I is negative (-0.65 and -0.35 respectively), showing an inverse correlation between these two variables as shown in the scatter plots below (Figure 11).

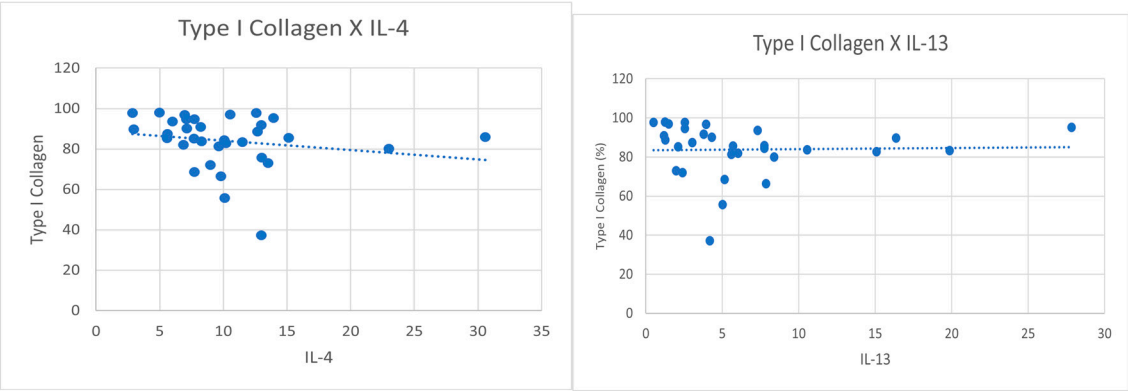

Figure 7. Left: Scatter plot showing a direct correlation between type I collagen and IL-4. Right: Scatter plot showing a direct correlation between collagen type I and IL-13.

The correlation coefficient between type III collagen and IL-13 and type III collagen and IL-4 is positive (0.35 and 0.65 respectively), showing a direct correlation between these two variables, as shown in the scatter diagrams below (Figure 12).

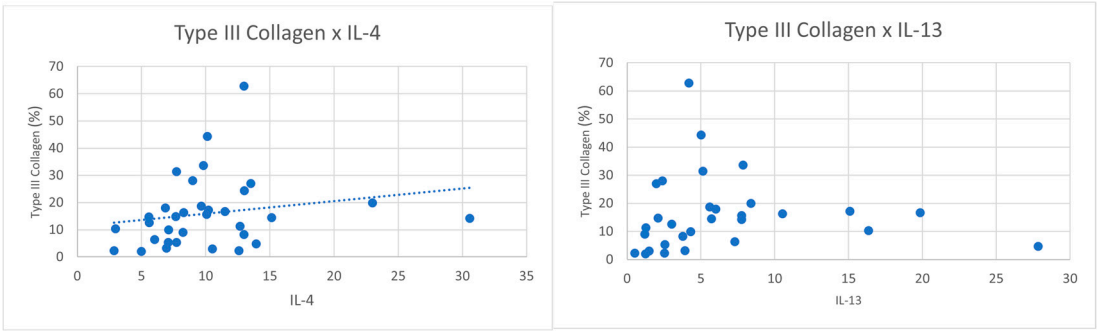

Figure 8. Left: Scatter plot showing a direct correlation between type III collagen and IL-4. Right: Scatter plot showing a direct correlation between collagen type III and IL-13.

4. Discussion

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In this study, at the end of 4 weeks of treatment, there was no significant difference across groups for presence of type I and III collagen (assessed by *Picrosirius Red*) or immunoexpression of IL-6.

On histological assessment of type I and III collagen fibers, the average percentage per group ranged from 80.3 to 88.4 for type I collagen and from 11.6 to 19.7 for type III collagen. Thus, all groups had a cellular matrix structure composed of >80% type I collagen fibers.

The absence of statistical differences for the presence of IL-6 at 4 weeks may be related to the fact that it is an acute-phase cytokine, so its correlation with collagen formation and tenocyte activity would be present in only in the first days following injury [24]. IL-6 peaks are seen between 3 days and up to 2 weeks after tendon injury; its elevated expression for longer periods is associated with extracellular matrix dysfunction by triggering a chronic positive feedback loop with interferon-gamma (IFN-g), the extracellular matrix-degrading interleukin [25,26].

As for the late-phase cytokines, IL-4 and IL-13, both showed significant differences between the groups and were present in higher concentrations in the SC + AM group than in the control group [27].

IL-4 provides negative feedback to IL-6 and regulates the initial inflammatory response, thereby promoting effective tissue repair through the stimulation of tenocyte differentiation and proliferation, which supports tissue repair and organization. The balance between IL-4 and other immune mediators, such as IL-13 and IL-10, is crucial for successful tendon regeneration [19,20,28–30].

The beneficial effects of IL-4 depend on both the timing and the context of the healing process. In certain situations, particularly during aging, an impaired immune response involving IL-4 and M2 macrophages can result in poor healing outcomes [20,28,31,32].

The significant increase in IL-4 observed in the SC + AM group suggests that tendon repair in this group is enhanced by the documented stimulatory effect of IL-4 on tenocytes.

IL-13, which is activated by IL-4, promotes tissue remodeling and collagen production by fibroblasts. Both interleukins correlate positively with the presence of type III collagen—the first collagen subtype to appear during tissue repair—while also promoting tenocyte differentiation and proliferation. IL-13 binds to receptors on tenocytes, stimulating genes that regulate the cell cycle and the production of proteins involved in tendon maintenance [19,28,33].

In the present study, the group treated with SC + AM showed increased levels of IL-13, corresponding with elevated levels of its upstream regulator, IL-4. Both interleukins correlated positively with type III collagen, confirming their involvement in the tendon regeneration pathway and further suggesting that this treatment facilitated repair.

The abscence of histological differences across groups does not necessarily represent homogeneity, but probably a snapshot of a moment when the repair process had not yet progressed enough to exert morphologically visible differences. Considering the between-group differences in inflammatory markers observed, it is likely that if we were to continue the study and carry out a later histological assessment, we would have foun histological differences as well.

This is further corroborated by a previous study by our group, in which we assessed the biomechanical strength and histology of Achilles tendons in rats treated with stem cells and platelet-rich plasma, in wich despite no histological differences or changes in the presence of type I and III collagen between the groups, the platelet-rich plasma group showed better biomechanics, implicating molecular mechanisms in the repair process [34].

During tendon healing, IL-4 acts as a signal for IL-13, an effector cytokine that stimulates fibroblast production, initially increasing the production of type III collagen, which over time can mature into type I collagen, further organizing the extracellular matrix, a correlation viewed in this study.

In the process of tissue repair, the amniotic membrane provides a collagenous support framework and facilitates the adhesion of growth factors, promoting healing in various types of lesions, including corneal lesions, venous ulcers and skin burns [35,36]. The combination of amniotic membrane and mesenchymal stem cells has already proved effective in healing full-thickness skin

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defects in rats, a finding that encouraged us to test this combination in the treatment of tendon injury [37].

Continued study inflammatory pathways should yeal potential targets for specific,effective treatment of inflammatory and other chronic diseases., just as blocking IL-21 is effective in controlling Rheumatoid Arthritis [35].

This study presents some limitations. The use of an animal model may be associated with physiological and anatomical differences between rats and humans, which can limit the direct extrapolation of the results. Moreover, the healing process in rodents tends to occur more rapidly than in humans. In addition, given the restricted sample size, the findings of this study should be considered preliminary, and further investigations with larger cohorts are recommended.

5. Conclusions

The molecular mechanisms underlying the pathogenesis of tendinopathies precede histological or morphological changes, making them a promising target for therapeutic intervention, as has already been demonstrated in diseases such as rheumatoid arthritis.

In chronic tendinopathy, low levels of IL-4 and IL-13 are associated with persistent inflammation and impaired tissue repair. In contrast, rats treated with stem cells and amniotic membrane exhibited higher levels of these interleukins, suggesting an immunohistochemical profile favorable to recovery and highlighting potential therapeutic strategies.

Local administration of IL-4 or IL-13 may represent a beneficial approach for accelerating tendon healing without compromising systemic immunity.

Ongoing and future studies may demonstrate that IL-4 and/or IL-13 represent promising therapeutic targets for tendon injuries, due to their ability to promote tenocyte proliferation and differentiation. These beneficial effects could be further enhanced through the use of scaffolds, such as the amniotic membrane, which support cell growth and the reconstruction of various tissues, including tendons.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Institutional Review Board Statement: All procedures involving humans and animals are in accordance with international ethical parameters (*ARRIVE guidelines*) and in accordance with the Local Ethics Committee (Comitê de Ética no Uso de Animais da Pontifícia Universidade Católica do Paraná, CEUA-Pr 01637, date 26/06/2019). An informed consent form was also obtained from the parturients who donated their placentas, in accordance with the Human Ethics Committee of the Victor Ferreira do Amaral Maternity Hospital (registration 01238, 02/08/2018).

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Data Availability Statement: The data collected during the study is available in the article and can be accessed in supplementary information files.

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Appendix A

Picrosirius Red data analysis

Grupo	Animal	% Col. type I	% Col. type III
SC	1	88,67228	11,32772
	2	94,68957	5,310428
	3	94,67009	5,329908
	4	71,99287	28,00713
	5	96,78912	3,210885
	6	97,76461	2,235387
	7	85,19644	14,80356
	8	91,76752	8,232476
	9	83,73942	16,26058
	10	37,24898	62,75102
AM +ST	1	95,25195	4,748048
	2	75,68604	24,31396
	3	83,30736	16,69264
	4	-	-
	5	85,80346	14,19654
	6	66,34731	33,65269
	7	-	-
	8	82,80798	17,19202
	9	81,30873	18,69127
	10	85,55754	14,44246
AM	1	93,74026	6,259735
	2	84,39343	15,60657
	3	82,02189	17,97811
	4	80,07587	19,92413
	5	90,08762	9,91238
	6	87,41478	12,58522
	7	73,04669	26,95331
	8	66,41677	33,58323
	9	55,63869	44,36131
	10	89,67368	10,32632
C (Control)	1	68,02493	31,97508
	2	97,72878	2,271217
	3	68,59478	31,40522

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4	97,01947	2,980529
5	90,97798	9,022016
6	96,26597	3,734033
7	93,64437	6,355624
8	97,96367	2,036329
9	85,24229	14,75771
10	-	-

Appendix B

Immunohistochemical data analysis

Group	Animal	IL-4	IL-6	IL-13
Sc	1	12,6851	0,09289	1,26152
	2	7,08174	0,37515	
	3	7,71709		2,55785
	4	8,97251	0,05649	2,39723
	5	6,95885		3,93043
	6	12,5989	0,22803	2,54793
	7	7,68494		
	8	12,9734		3,78649
	9	8,27816	0,14316	10,5361
	10	12,9734	0,19234	4,18266
SC + AM	1	13,9265		27,8312
	2	13,0249	1,93656	
	3	11,5018		19,8547
	4	30,5643	0,25865	7,75376
	5			
	6	10,2099		15,0871
	7	9,64392	0,24008	5,59404
	8	15,1371	1,28579	5,70805
AM	1			
	2	10,0641	0,53462	7,75376
	3	6,86066		6,01953
	4	22,988	0,30739	8,3792
	5	7,11552	0,1392	4,30523
	6	5,60763	0,07892	3,03288
	7	13,5096	0,16221	1,98716
	8	9,81983	0,11303	7,86464
	9	10,1212	0,1319	5,00698
	10	2,97585	0,32952	16,3635
C	1			
	2	2,8693	0,15288	0,51087
	3	7,73207		5,13824

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4	10,5307	0,28865	1,49216
5	8,21829	0,69733	1,19455
6			
7	6,01073	0,63131	7,3027
8	4,98184		1,2504
9	5,5838		2,10399

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