Effects of Elastic High-Molecular Weight Poly (L-Co-D, L Lactic Acid) with 1,3-Trimethylene Carbonate Membrane in the Expression of Collagen and Glycosaminoglycans Tendon Healing, A Randomized Controlled Trial in Rabbits

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Abstract

Background: The use of membranes has been reported to present a positive impact on tendon healing. Thus refinements in absorbable materials and the best knowledge of the reparative process are important to drive clinicians into choose the more suitable therapeutic options.

Hypothesis/Purpose: Membranes presenting low crystallinity within an elastic structure may result in more biocompatible materials, improving healing at the same time that minimizes side effects related to the absorbable materials.

Study Design: It is a preclinical prospective randomized case-control study using animal model (New Zealand rabbits).

Methods: This study assessed the effectiveness of a new absorbable membrane that possesses mechanical and chemical features more suitable to tendons. The 46 rabbits underwent tenotomy and reconstruction of the right gastrocnemius tendons. They were randomly divided into three groups: case group (tendons wrapped with a membrane), control group, and intact tendon group. Animals of both the groups were allocated for extraction at seven, 14 and 28 days. Macroscopy, glycosaminoglycans, type I collagen (through polarized light) and histologic assessments were performed. Statistical tests respected the nature of the data in an adaptive model.

Results: At 14 and 28 days, the tendons of the case group presented less adherences (p=0.02 and p=0.03, respectively). Glycosaminoglycans (GAGs) presented statistical differences only for the central part of the tendon for Dermatan Sulfate and Hyaluronic Acid, both for the 7th day assessment p=0.02 and 0.04 respectively. Chondroitin Sulfate in the same part of the tendon presented a difference p=0.05. At 14 days, there was equivalence between the samples for CS and DS p>0.39. The modified histologic Watkins classification for case and control groups averages: 14.67 ± 0.42 and 12.67 ± 0.56 (p=0.03) at 14 days, and 19.88 ± 0.83 and 17.25 ± 0.62 (p=0.02) at 28 days. The mean of gray value for codified red color on Image-J® for type I collagen were 2.41 ± 0.33 and 1.31 ± 0.18 (p<0.0001) at 28 days.

Conclusion: These results suggest that the tendons wrapped with the new membrane presented changes that facilitated the healing process of the gastrocnemius tendons of New Zealand rabbits.

Keywords: Tendon healing; Absorbable implants; Tendon injuries; Glycosaminoglycan; Proteoglycan

What is known about this Subject?

Intact tendons possess low cellularity, and hence, have low capability to heal [1]. Tendons are...
organized structures with high resistance, mainly to traction, due to the high density of type I collagen within its structure [2]. The perfect combination of resistance and elasticity enables tendons to dissipate energy to prevent lesions [3]. The risk of re-injury in tendons is high, even after initial successful repair [4-8]. Tendon healing, which changes the mechanical and biological properties, is a long process that may present definitive fibrotic modifications within the tendon structure.

Adhesions between the tendon and surrounding tissues may impede the normal excursion of the tendon [9,10], compromising the correct mechanical stimulus and thus creating structures less resistant to traction than before [11]. Unsuccessful repairs seem to present a direct correlation with these structural changes at the site of tendon lesion [12,13]. Biomechanical studies have suggested that tendon re-tears are more likely to occur just adjacent to the previous injury site [14]. The pathogen of tendons is not completely elucidated; however, extracellular matrix synthesis is abnormal and inflammation, even transient, is present [15-17].

In tendinopathy, all tendons are affected, exhibiting changes in their biological and mechanical features [9]. The extracellular matrix of tendons predominantly consists of, hyaluronic acid proteoglycans such as decorin, biglycan, fibromodulin, lumican, aggrecan, and versican, which can regulate the production of type I collagen [11]. An overall increase in proteoglycans and their sulfated Glycosaminoglycan (GAG) side chains is observed in the space surrounding the collagen fibers in lesioned tendons [18]. However, not all proteoglycans or GAGs are changed in tendinopathy. The levels of decorin and keratan sulfate appear to be unchanged in pathologic tendons, in contrast to biglycan, versican and aggrecan [19-23]. GAGs, such as Chondroitin Sulfate (CS) and Dermatan Sulfate (DS), are highly expressed even in tendons presenting pathologic conditions [24,21,12]. Increased rates of GAGs are also associated with tendon pain increased tendon cross-sectional diameter and impaired collagen structure [25-8]. Biomembranes have been demonstrated to improve tendon healing, facilitating their reparative processes [29]. Less adherences and the possibility of earlier and better mechanical stimulus to the repaired tendon can result in the successful use of biomembranes [10].

**What this Study Adds to Existing Knowledge?**

A bioabsorbable membrane was created using Poly (L-co-D, L lactic acid) (PLDLA) with 1,3-Trimethylene Carbonate (TMC) of high molecular weight. The combination of these materials results in the most elastic, less crystalline, and quick absorption membrane. These characteristics make this membrane more biocompatible, potentially allowing a better reparative capability. In this study, the GAGs and the histological and macroscopic features of tendons were investigated at three different time points to determine the effectiveness of the bioabsorbable membrane on tendon healing.

**Materials and Methods**

**Study design**

It is a preclinical prospective randomized blinded case-control study using animal model (New Zealand rabbits).

**Set for data collection**

Surgical pathophysiology laboratory of the University which the author is affiliated.

**Polymer synthesis**

The copolymers L and D,L-lactate were prepared using a mass polymerization reaction in a glass ampoule. The ratio of the monomers L-lactate (Sigma-Aldrich, Brazil) and D,L-lactate (Sigma-Aldrich, Brazil) for the syntheses was 70:30. The catalyst was tin (II) 2-ethylhexanoate, better known as Sn (Oct) 2.

**The monomer:** Catalyst ratio was approximately 5000, optimizing the production of high molecular mass PLDLA [30]. The monomers (L-lactate and D,L-lactate) and the catalyst were inserted into the glass ampoule with TMc in a new 70:30 proportion. The glass ampoule was sealed under vacuum and immersed in an oil bath at 130°C for 72 h. Subsequently, the polymer was dissolved in chloroform (CHCl3; Merck Millipore, Germany) and precipitated in methanol (CH3OH; Merck Millipore, Germany). The PLDLA-TMC (70:30) membrane was made using the casting method and dried in a desiccator with a vacuum at 45°C for 8 h.

**Animals and surgical procedure**

The membranes were biologically tested in New Zealand white rabbits weighing 2000 ± 200 g with 3 months old. The rabbits were kept in a circadian cycle under controlled conditions. They were randomly divided as follows. Nine animals were used as positive control for GAGs; this group was named as the intact tendon group. All other animals underwent tenotomy of the right medial and lateral gastrocnemius tendon. Tenotomy was performed 2 cm proximal to its insertion on the bone. The rabbits underwent tendon reconstruction by Kessler suture using FiberWire 2 (Arthrex, FL, USA).

Two groups of six rabbits at each study time point were used. The rabbits were assessed at 7, 14, and 28 days to investigate the membrane in different phases of healing process. The adaptive statistical model required two more rabbits for each group for the assessment at 28 days, total of 40 rabbits for all study [31]. The soleus muscle was left intact. A plastic orthosis (Figure1) fixed with bands was used during the first two weeks to improve tendon protection at the initial healing phase. The orthosis did not impede, but restricted the movements of the rabbits. The tendons of the rabbits from the case group were wrapped using the new membrane. The membrane was fixed to the tendon with two 4.0 nylon sutures. All tendons were macroscopically assessed and extracted 0.5 cm to 3.5 cm from the calcaneus bone. A third group of six rabbits was used to extract intact tendons.

**Macroscopy**

The parameters used for the macroscopic assessment were as follows: Adherence (absent, minimal, mild, moderate, or high), continuity (organized or unorganized), and repair (absent or present). The tendons were horizontally split into two parts. One part was vertically subdivided into three subgroups for GAG analysis: Proximal, central (in the lesion site), and distal. The other part was used for histologic assessment. Collagen and fibers were not assessed in the 7-day groups because changes in their expressions occur at earlier stages of healing.

**GAG quantification**

The three subdivisions of each tendon were separately assessed CS, DS, and Hyaluronic Acid (HA) were measured. An additional positive control group comprising six rabbits was also used to determine the normal tendon patterns of GAGs. The samples were pulverized using scissors and directly macerated. Peptides and nucleic acid fragments were removed by precipitation with 10% trichloroacetic acid at 4°C. After centrifugation (10 min, 3500 x g, 4°C), the supernatant containing GAGs was precipitated by adding...
two volumes of methanol for 18 h at 4°C. The precipitate was separated and collected through centrifugation (10 min, 3500 x g, 4°C) and then dried. Subsequently, it was suspended in 40 ml of distilled water and analyzed for sulfated GAG. This method of recovering GAGs extracted from the tendons gives a yield of approximately 95% [32]. Sulfated GAGs were identified and quantified with agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0. An aliquot of 5 ml of each sample was submitted to electrophoresis for 1 h at 100 V; GAGs were precipitated in the gel with 0.1% cetyltrimethylammonium bromide for 2 h at room temperature. The gel was dried and stained with a 0.1% solution of toluidine blue in acetic acid ethanol and water (0.1:5:4.9, vol/vol). Quantification was carried out by densitometry at 530 nm of the toluidine blue-stained electrophoretic slide. The extinction coefficients of the GAGs were calculated using the standards of CS and DS. Identification of the sulfated GAGs was based on the migration of the compounds compared with those of the standards. The identities of GAGs present in the samples were further confirmed by treatment with specific enzymes. Electrophoresis was performed in triplicate. Results of the absolute amounts of GAGs were expressed per weight of tendon (µg mg⁻¹) [33-35].

HA was quantified by fluorometric noncompetitive Enzyme-Linked Immunosorbent Assay - Like Fluorescence Assays (ELISA) that can detect 2e 500 g/L of HA. The ELISA plates had a fixed probe, and 100 ml well of standard HA solutions at various concentrations (0e500 g/L) was added. The samples were diluted (1:100) in a Tris hydrochloride buffer 0.05 M with 1% bovine serum albumin and added to the ELISA plates in triplicate. The plates were incubated at 4°C for 12 h and then washed three times with Tris hydrochloride 0.05 M. Afterward, 100 ml of the probe (1 mg/ml) diluted (1:10,000) in the assay buffer was added. The plate was incubated for 2 h on a shaker and washed nine times with a wash buffer. Streptavidin labeled with europium diluted to 1:10,000 in assay buffer was added to each well (100 ml; Sigma, Germany). Streptavidin has an affinity for the biotin-conjugated probe. The plate was incubated 30 min on a shaker and washed nine times with Tris hydrochloride 0.05 M. To release the europium bound to streptavidin (Sigma), an enhancement solution (280 ml/well) was added. The plates were agitated for 5 min, and the europium-free plate fluorescence was read on a fluorimeter. The results were expressed in ng/ml [36].

Histology

The colorations used for the assessments were eosin-hematoxylin, Masson’s trichrome and picrosirius. To evaluate tendon healing, the author modified the tendon maturing scoring system reported by Watkins et al. [37] (Table 1). Six histologic parameters including cellularity, fibrocytes, vascularity, fiber diameter, cells parallel, and fibers parallel were evaluated. One more category, i.e., collagen polarized light assessment colored with picrosirius red, was added to evaluate the type I/type III collagen ratio. Values between 7 and 28 points can be achieved using this score healing process. The reason for this scoring is that type III collagen first increases and then decreases, followed by its replacement with type I collagen, during the process of tendon healing. The proportion of type I/type III collagen has therefore a direct correlation with better mechanical properties of tendon healing [38-40]. The samples were scored in a blinded manner. The author also performed an objective assessment of type I collagen colored with picrosirius red by using polarized light. The assessment was conducted by photographing five different fields, within the regeneration site per sample, and Image-J (National Institutes of Health, USA) was then used to divide each photo into three different colors and separate the red color only, process it to gray and thereafter quantify the Mean Gray Value (Mgv). It is the sum of the gray values of all pixels in the selection divided by the number of pixels.

The fields for the photos were chosen in a blinded manner at 400x amplification, 1445250 (1388×1040) pixels. Each field was considered a different sample for statistical purposes. This was the primary outcome for 14 and 28 days.

Statistics

Statistical tests were performed according to the nature of the data.

Randomization was performed by using the App Randomization Lite® (The Apps Factory-USA). The Rabbits received new random number after the tissue extraction in order to random the allocation using the same App mentioned above.

All assessments were blinded and conducted using Prism7 for Mac OS X (GraphPad, CA, USA) STATA (STATA Corp®, TX, USA) and Wizard 1.9.7 for Mac (Free Software Foundation-USA). A two-tailed p<0.05 was considered significant.

An adaptive statistical method was performed to optimize the sample size after an initial interim analysis based on six animals per group, considering an alpha of 0.05 within a statistical power of 90% [31].

The sample size was reached based on the primary outcomes for each period of assessment through the interim analysis.

For 14 and 28 days the primary outcome was the objective measure of Type I collagen, with an expected effect of at least 1.00 mg/v for 14 days and 1.50 mg/v for 28 days.

For 7 and 14 days the initial sample sizes were suitable, however for 28 days two more rabbits were required for each group.

Data will be described using mean ± standard error.

Results

Macroscopy

At 7 days none of the animals’ tendons presented healing features that allowed them to be assessed by the standardized macroscopic parameters. They presented gelatinous to liquid consistency, markedly in its central part of the case group.

Adherences were different at 14 days (p=0.02) and 28 days (p=0.03) (Figure 2). Results are summarized in Charts 1 and 2. With regard to continuity, the assessment presented a difference favorable to the case group at 14 days (p=0.04) and 28 days (p=0.13). Repair was...
observed in all samples at 14 and 28 days.

**Glycosaminoglycan levels**

Glycosaminoglycan measurements are shown in Table 2. When differences were not observed in the case and control groups at 14 days. Measurement was not performed at 28 days because in 14 days an equivalence between case and control have already been established for \( P > 0.39 \). HA was just measured for seven days because of technical problems.

**Microscopy**

None of the animals presented healing features that allowed them to be evaluated at 7 days. According to the modified Watkins classification, the case and control groups presented averages of 14.67 ± 0.42 and 12.67 ± 0.56, respectively, \( P = 0.03 \) at 14 days. At 28 days, the case group presented an average of 19.88 ± 0.83 and the control group an average of 17.25 ± 0.62 (\( P = 0.02 \)).

The quantification of type I collagen by polarized light showed the following results. At 14 days, averages of 2.41 ± 0.33 mgv for the case group and 1.31 ± 0.18 mgv for the control group (\( P = 0.01 \)) were observed (Figure 3). At 28 days, averages of 7.30 ± 0.63 mgv for the case group and 2.92 ± 0.32 mgv for the control group (\( P < 0.0001 \)) was obtained (Figure 4). The intact tendon presented an average of 41.10 ± 0.32 mgv (Figure 5).

**Discussion**

The membranes used in this study were effective in preventing adherences, which is similar to the reports in literature [10,29]. However, other membranes are non-absorbable or when absorbable, their long absorption time can negatively affect the healing process [41]. The low crystallinity of this study’s copolymer makes it more biocompatible [30,42].

The use of TMC provided additional elasticity, which is favorable in achieving a better fit to the tendon with less interference within the tendon’s energy transmission. Some studies suggested that CS and DS concentrations are higher when the pathologic conditions of the tendon are considered [12,21,24]. This finding was confirmed for CS in the present study. However, the DS in normal tendons was higher than that ones for all groups. Assessments of CS at 7 days showed a statistically significant difference for distal samples favorable to the

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Table 2: Results GAGs 7 days, 14 days and Intact Tendon.

<table>
<thead>
<tr>
<th></th>
<th>7 days</th>
<th>14 days</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Central</td>
<td>Distal</td>
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<tr>
<td>Chondroitin case</td>
<td>0.48 ± 0.05</td>
<td>0.44 ± 0.77</td>
<td>0.80 ± 0.04</td>
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<tr>
<td>Chondroitin control</td>
<td>0.31 ± 0.08</td>
<td>0.69 ± 0.09</td>
<td>0.38 ± 0.04</td>
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<tr>
<td>P</td>
<td>0.0084</td>
<td>0.05</td>
<td>0.029</td>
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<td>Dermatan case</td>
<td>1.21 ± 0.11</td>
<td>0.42 ± 0.09</td>
<td>1.48 ± 0.20</td>
</tr>
<tr>
<td>Dermatan control</td>
<td>0.90 ± 0.10</td>
<td>1.29 ± 0.67</td>
<td>1.17 ± 0.13</td>
</tr>
<tr>
<td>P</td>
<td>0.07</td>
<td>0.02</td>
<td>0.23</td>
</tr>
<tr>
<td>Hyaluronic Acid case</td>
<td>9.98 ± 2.79</td>
<td>17.72 ± 3.62</td>
<td>10.65 ± 3.13</td>
</tr>
<tr>
<td>Hyaluronic Acid control</td>
<td>8.52 ± 2.11</td>
<td>9.48 ± 1.61</td>
<td>10.91 ± 3.86</td>
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<tr>
<td>P</td>
<td>1</td>
<td>0.04</td>
<td>0.94</td>
</tr>
<tr>
<td>Intact Tendon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin</td>
<td>0.67 ± 0.21</td>
<td>1.01 ± 0.13</td>
<td>0.65 ± 0.09</td>
</tr>
<tr>
<td>control</td>
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<tr>
<td>P</td>
<td>0.994</td>
<td>0.394</td>
<td>0.802</td>
</tr>
<tr>
<td>Dermatan case</td>
<td>1.20 ± 0.13</td>
<td>1.39 ± 0.22</td>
<td>1.11 ± 0.05</td>
</tr>
<tr>
<td>Dermatan control</td>
<td>1.08 ± 0.23</td>
<td>1.16 ± 0.38</td>
<td>1.27 ± 0.12</td>
</tr>
<tr>
<td>P</td>
<td>0.66</td>
<td>0.58</td>
<td>0.87</td>
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Figure 1: Plastic orthosis.

Figure 2: Macroscopy.

Figure 3: Type I Collagen 14 days: Control (right) and Case (left).

Figure 4: Type I Collagen 28 days: Control (right) and Case (left).

Figure 5: Type I Collagen Intact Tendon.
case group, a difference for proximal samples favorable to the case group, and a difference for central samples favorable to the control group. Assessments of CS at 14 days showed equivalence for all samples. These data demonstrate that CS was elevated in all areas, which contributed to the healing process. These data, associated posterior macroscopic and histologic findings, contribute to suggest that within the earlier phases of the healing process the GAGs levels can be affected by the membrane using, accelerating healing.

The membrane seemed to cause delay in the elevation of chondroitin concentration for the central part of the tendon, which improved in the following week, and an elevation on other parts during the first week?

The assessment at 7 days showed differences on DS concentrations between the groups favorable to the case group at the proximal tendon, statistically significant difference favorable to the control group in the central part of the tendon, and no difference at the distal tendon.

The liquid consistency, markedly within the tendon’s central portion for the case group assessed at 7 days and a statistical difference on HA concentrations suggest that this hydrophilic substance has an important role during the very early healing process, as mentioned by other authors [43,44].

DS demonstrated equivalence between the case and control groups in the assessment at 14 days.

The membrane seemed to change the sulfated GAGs concentrations in all tendon areas for the first stages of the healing processes.

Some studies associated higher sulfated GAGs to inferior tendon biomechanics in the long term [18]. However, Özer et al. [44] suggested an improvement in tendon healing for rats treated with oral glucosamine and CS. Other authors found elevations on CS levels during the healing process for ligaments [46].

In the present study, healing process seems also to present a positive correlation with CS levels in short term assessments for tendons.

In this study, the DS level was lower in pathologic tendons than that in normal tendons at any time point, but the CS level was elevated; CS was not even present in intact tendons. Thus CS appears to have an important role in the early stages of healing process but not for mature tendons [45]. The modified Watkins [37] classification demonstrated that healing process was superior in the case group at 14 and 28 days. Tendon healing was also improved in other situations, such as tendon bone healing assisted by absorbable membranes [47].

With regard to the objective data from the polarized light measurement, an improvement of type I collagen at 14 and 28 days in the case groups was observed. The 28-day improvement established a definitive difference between the groups according to the Haybittle-Peto boundary [48]. Fewer adherences seem to be the key to achieve accelerated healing. It allows early mechanical stimulus. Once the mechanical stimulus is associated with better healing results and the energy direction is better canalized using the membrane the force lines will be better oriented, mitigating radial dissipation through the scars [49,50].

**Limitations**

This is a preclinical trial; therefore clinical trials are necessary to understand whether the good results presented in this study can be replicated for humans.

Macroscopic assessments cannot be blinded with possible bias for this outcome.

The absence of detectable harms for the animals enrolled does not warrant that harms will not take place for animals nor humans.

**Conclusion**

The absorbable membrane promoted early and better mechanical stimulus within more balanced responses, accelerating the healing process by mitigating radial energy dissipation through the scars. It also presented a short-term degradation that mitigated any long-term complications that absorbable devices can promote. The presented membrane could change the GAGs and histological structure, accelerating calcaneal tendon healing in New Zealand rabbits.

**References**


