Effects of Human Placenta Extract (Laennec) on Ligament Healing in a Rodent Model

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Rich in bioactive substances such as amino acids and peptides, Laennec (human placenta hydrolysate) has been widely used to control various types of musculoskeletal pain. However, the effects of Laennec on tendon and ligament injuries are not clearly understood. In the present study, Laennec was tested to identify its in vivo effects on ligament injury in an animal model and its in vitro effects on tendon-derived fibrocytes. A total of 99 Sprague Dawley rats were divided into the negative control (normal) group (n = 11) and the ligament injury group (n = 88). The ligament injury group was subdivided into normal saline-treated group, Laennec-treated group, polydeoxyribonucleotide-treated group, and 20% dextrose-treated group. Ligaments were collected at 1 week and 4 weeks after treatment. Histologic and biomechanical properties were analyzed. In vitro effects of Laennec and polydeoxyribonucleotide on fibrocytes were also analyzed. Although all other treatment groups showed increased inflammatory cells, the Laennec-treated group maintained cell counts and activated macrophage levels that were similar to the normal group. Unlike the saline-treated group and dextrose-treated group, the Laennec-treated group had low levels of degenerative changes at 4 weeks after treatment. Supportively, in vitro results showed that the Laennec-treated group had increased collagen type I, scleraxis (Sex) and tenomodulin (Tnmd) expression (p < 0.05). Our study demonstrates that Laennec treatment enhances wound healing of damaged ligament by suppressing immune responses and reducing degenerative changes of damaged ligament. In addition, we found that Laennec induces the gene expression of type I collagen, Sex and Tnmd in fibrocytes, suggesting that Laennec may facilitate regeneration of damaged ligaments. Therefore, we expect that Laennec can be a useful drug to treat injured ligament.

Key words human placenta hydrolysate; Laennec; prolotherapy; ligament; tendon

INTRODUCTION

The increased popularity of sports and leisure activities has led to more injuries involving tendons or ligaments. Acute tendon/ligament injuries include sprains and partial and complete tears, and approximately 30–50% of such injuries are associated with exercise. The basic method of treating acute tendon/ligament injuries involves promoting natural healing by initial immobilization followed by rehabilitation exercises. Clinically, a sprained ligament requires approximately 2–4 weeks to heal, and a partial tear requires approximately 4–12 weeks to heal. However, pain may persist even after treatment, and the damaged tendon or ligament may be reinjured before complete recovery, which can cause an inflammatory response and become a long-term problem. These old ligament problems are usually referred to as tendinopathy.

Since there is no definitive treatment for acute or chronic tendon/ligament injuries, various types of regenerative treatments are used as alternative methods in clinical practice. Clinical treatments for acute or chronic tendon/ligament injuries include prolotherapy, extracorporeal shockwave therapy (ESWT), low-intensity ultrasonography (LIUS), and platelet-rich plasma (PRP) injection therapy. However, most of these methods lack evidence regarding their therapeutic efficacy.

Human placenta hydrolysate (hPH) such as Laennec is used to improve liver function in patients with chronic liver disease, however, it is being used in a variety of ways in clinical practice, from wound healing to immunoregulation. According to recent studies, Laennec is rich in bioactive substances, such as peptides and amino acids. Recently, it has been used as a prolotherapy to treat acute tendon or ligament injury in the musculoskeletal system. Therefore, in the present study, we examined the regenerative effects of Laennec in ligament using in vitro and in vivo system in an artificial ligament injury model.

MATERIALS AND METHODS

Animal Model of Medial Collateral Ligament Injury

The animal protocol used in this study was approved by the Inha University Institutional Animal Care and Use Committee (Inha IACUC) and was in accordance with their ethical procedures and scientific care (Inha IACUC 160928-440-1). Rats were maintained in air-filtered cages and fed normal mouse chow at the Inha University Hospital Animal Care Facility. An animal model of subfailure ligament injury was created using the medial collateral ligament (MCL) of Sprague Dawley rats. Male Sprague Dawley rats (approximate weight, 280–300 g) were anesthetized by an intraperitoneal (i.p.) injection of 40 mg/kg ketamine and 10 mg/kg xylazine hydrochlo-
ride. A longitudinal skin incision was made on the medial aspect of the both knee. After identifying the MCL, a modified paper clip was passed beneath the MCL, between the ligament and the knee joint. The clip was bent so that it would hook underneath the MCL. The other end of the clip was connected to the hook of the push-pull gauge fixed on a stand. A custom-made leg-holding plate was used to keep the animal stable on the base while traction force was applied to the MCL. Continuous traction force of 10 N was applied to the MCL for 5 min to create a partial tear injury. The severity of the ligament injury was identified using an electron microscope (Fig. 2).

**Experimental Design** A total of 99 Sprague Dawley rats (8-week-old males; Orient Bio, Seongnam, Gyeonggi-do Province, South Korea) were used to create the models and comprised the normal and ligament injury groups. A total of 11 rats were assigned to the negative control (normal) group and 88 rats were assigned to the ligament injury groups (Table 1). In the ligament injury group, a predetermined medication was administered immediately after the injury and 3, 7, 14, and 21 d after the injury. We tested and determined the maximum injectable volume in the injured area according to previous studies. The administered medicine consisted of 1 mL of normal saline, 1 mL of Laennec (Laennec injection; 3.6 mL/kg; 30632; Green Cross WellBeing, Gyeonggi-do, Korea), 1 mL of polydeoxyribonucleotide (PDRN) (Placentex injection; 1.875 mg/mL; #910058; Pharma Research Products), and 1 mL of 20% dextrose (20% glucose solution; #16005; JW Pharmaceutical, Seoul, South Korea) (Fig. 2). The PDRN is used for ligament injuries in South Korea, dextrose is used for the prolotherapy. The MCL of the rat was palpated and medication was injected near the ligament so that it was spread evenly around the ligament.

**Assessment**

**Histologic Analysis**

Rats were euthanized using CO₂ inhalation, and MCL samples were collected at 1 week (n = 55) and 4 weeks (n = 44) after injury. The 11 samples were analyzed per group in all kind of analysis. All samples were fixed in 4% paraformaldehyde and embedded in paraffin. The 4-µm-thick sections were obtained and stained with standardized hematoxylin and eosin (H&E) and Masson’s trichrome. Immunostaining was performed using mouse anti-rat CD68 (ED1) for activated microglia and macrophages (1:100; AbDSerotec, Kidlington, U.K.). The primary antibody was visualized with rhodamine-conjugated donkey anti-mouse immunoglobulin G (IgG) (H+L) (1:500; Jackson ImmunoResearch, West Grove, PA, U.S.A.). All sections were mounted with 4’,6-diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories, Burlingame, CA, U.S.A.).

To assess the injury level, a modified Bonar scale was used to evaluate sections stained with H&E and Masson’s trichrome. The injury level was categorized according to cellularity, cell shape, and collagen fiber alignment and was rated using a score of 1 to 3 (1, minimal change; 2, moderate change; 3, severe change).

**Table 1. Experimental Design and Outcomes of the Animal Model Using Laennec**

| Groups                  | Euthanized at 1 week | Euthanized at 4 weeks |
|-------------------------|----------------------|-----------------------|
| Normal group            | 22                   | 22                    |
| Ligament injury group   |                      |                       |
| Normal saline           | 22                   | 22                    |
| Laennec                 | 22                   | 22                    |
| PDRN                    | 22                   | 22                    |
| 20% Dextrose            | 22                   | 22                    |
change; 3, severe change). The modified Bonar scale scores were expressed as the sum of three categories. All histologic slides were scored by three investigators who were blinded to the group identity of the specimens.

To assess inflammation, the infiltrated cells and activated macrophages were measured by DAPI and EDI staining. DAPI-stained nuclei were measured in three regions on tissue sections (Image J). We counted DAPI positive cells with round shape of nuclei using modified Bonar scale analysis to indicate infiltrated cells, which may be represented as inflammatory cells. The mean values of DAPI at three regions were measured. An activated macrophage marker, CD68 (EDI), was measured by the fluorescence intensity to measure the infiltrated macrophages with corrected total cell fluorescence (CTCF) using Image J (National Institutes of Health, Bethesda, MD, U.S.A.).

All histological samples were examined using a Zeiss Axiosplan2 (Oberkochen, Germany) up-light fluorescence microscope with a SPOT flex camera (SPOT Imaging, Sterling Heights, MI, U.S.A.).

Biomechanical Tensile Testing

The injured MCL was harvested to undergo biomechanical testing. The width and thickness of the MCL ligament were measured with digital calipers, and the estimated cross-sectional areas were calculated with elliptical geometry. The ligament was fixed to a universal testing machine (ElectroForce5500; BOSE, Eden Prairie, MN, U.S.A.) using a custom-made jig and clamp. Each end of the ligament was clamped between two pieces of sandpaper in a tissue grip to prevent slipping. The exact original length (L0) between the mounting jigs was measured at the time of the onset of force. After 10 cycles of preconditioning, ligaments were pulled to failure using controlled displacement at a constant speed (0.2 mm/s). Force and displacement data were collected at 200 Hz using WinTest 7 software (ElectroForce, BOSE, Eden Prairie, MN, U.S.A.). The ultimate tensile region to the beginning of the yield point of the stress-strain curve was measured for each specimen.

Efficacy of Laennec at the Cell Level Using an In Vitro Assay with Ligament-Derived Fibroblast Culture

To assess the effects of Laennec, MCL explants were seeded and tendon-specific ECM gene regulator and collagen type 1 expressions was measured. Briefly, after harvesting the MCL of the animal, the lateral collateral ligament was cut into small pieces. Each segment was placed in 60-mm culture dishes containing 3 mL of growth medium. Growth medium consisted of Eagle’s minimum essential medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal bovine serum (ATCC, Manassas, VA, U.S.A.), non-essential amino acid, l-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 µg/mL). Culture dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂. Culture medium was changed every 2–3 d, and cell growth in the explants was monitored. After 10 d, when cultured cells filled 70–80% of the culture dish, explants were removed and ligament-derived fibroblasts were detached with trypsin/ethylenediaminetraacetic acid (EDTA) (Gibco) for the subculture. Laennec or PDRN was added to the cultured fibroblasts so that the volume was approximately 5% of the medium. After 24 h in culture, expression levels of collagen type I (COL1), scleraxis (Scx), and tenomodulin (Tnmd) were identified by RT-PCR. Total RNA was extracted using TRIzol reagent (15596026; Thermofisher, Waltham, MA, U.S.A.).

cDNA was synthesized from 1 µg of total RNA using the AccuPower cDNA synthesis kit (Bioneer, Daejeon, South Korea). The primer sequences were as follows: rat COL1, forward 5'-CAACAATACTCCCACAAC-3' and reverse 5'-CACACAAGACAAGACACGAG-3'; Scx, forward 5'-GACGCACCGACACACAGTGTA-3' and reverse 5'-GTGACCCCTCTCTCTCTCTCTTACTCT-3'; TNMD, forward 5'-TTGAGAGACCACGAAATGAAGATGA-3' and reverse 5'-ATGGACACCTGGAGCAGACCACTTCTCTC-3'; and 18S RNA, forward 5'-TGGCAGAAGATCTATTACACGGA-3' and reverse 5'-GCAATTATTTCTGCTGAAAGCG-3'.

Statistical Analysis

All statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, version 22.0; IBM Corp., Armonk, NY, U.S.A.). The chi-square test was used to determine the results of the semi-quantitative assessment of ligament injury using H&E staining. One way ANOVA test was performed for the Bonar scale, followed by a posttest using the Tukey’s honestly significant difference (HSD). To determine the number of infiltrated cells using DAPI and EDI, a Kruskal–Wallis analysis was performed, followed by a posttest using the least significant difference (LSD). During the analysis of the ultimate tensile strength (UTS) and tensile modulus, also a Kruskal–Wallis analysis was performed, followed by a posttest using the LSD. For the in vitro fibrobyte assay, the Kruskal–Wallis test was performed, followed by a posttest using the LSD. All results are presented as the mean ± standard deviation (S.D.); p ≤ 0.05 was assumed to be statistically significant.

RESULTS

Histologic Analysis

Semi-quantitative Assessment Using the Modified Bonar Scale

When pathological findings of the ligament injury were compared using a semi-quantitative method and the modified Bonar scale (score of 3: ligament closest to being normal; score of 9: most severely damaged ligament), the results indicated that the group treated with saline (p = 0.034) and the group treated with dextrose (p = 0.022) had significantly higher injury scores than the normal group after 4 weeks, whereas the group treated with PDRN (p = 0.121) and the group treated with Laennec (p = 0.229) had scores that were not statistically significantly different compared to the group with no ligament injury (Fig. 3, Table 2).

DAPI Analysis to Evaluate the Number of Infiltrated Cells

After the ligament injury, the saline (p = 0.046) or the dextrose treatment (p < 0.01) shows significantly increased the number of infiltrated cells compared to the normal at 1 week. However, the Laennec or PDRN treatment maintains the number of cells similar to the normal ligament group at 1 week. Significantly, the Laennec treatment show no differences in the number of infiltrated cells compared to the normal at 4-week after treatment, while other groups including saline, dextrose, and PDRN treatment have still increased the number of infiltrated cells (p < 0.01) (Fig. 4).

CD68 (EDI) Immunostaining for Assessing Inflammation

The fluorescence intensity analysis after CD68 (EDI) staining showed increased macrophage density compared to the normal ligament at 1 week after the injury for all groups.
except the saline-treated group (Laennec, $p = 0.045$; PDRN, $p < 0.01$; dextrose, $p = 0.014$). The macrophage density was increased at 4 weeks after treatment with saline, dextrose, and PDRN compared to the normal ligament ($p < 0.01$). Significantly, Laennec treatment inhibited the increase of macrophage density and showed similar levels to the normal ligament (Fig. 5).

### Biomechanical Analysis

The tensile strength test of ligament samples collected 1 week after administration of saline, dextrose, PDRN, or Laennec after injury showed slightly lower UTS for the four groups than for the normal ligament group, but the differences were not statistically significant. Regarding the tensile modulus, the group treated with PDRN had a significantly higher tensile modulus than the normal ligament group at 1 week after injection ($p = 0.003$), which indicated that the tissue had lost its elasticity and become stiffer. The group treated with Laennec maintained levels of UTS and a tensile modulus similar to that of the normal ligament group. The tensile strength test of ligament samples collected 4 weeks after treatment with saline, dextrose, PDRN, or Laennec showed that the UTS and tensile modulus in all four groups were not significantly different compared to the normal ligament group. The group treated with Laennec showed similar levels of UTS and a tensile modulus to that of the normal ligament group at 4 weeks after treatment, indicating that Laennec can maintain the physical and functional characteristics similar to those of the normal ligament (Fig. 6).

### Effects of Laennec on Fibrocytes in Vitro

The group treated with Laennec showed increased expressions of collagen type I, Scx (a differentiation marker of the tendon cell), and Tnmd (tendon maturation), which are associated with ligament regeneration. However, the results of the Laennec-treated group were significantly different compared to those of the PDRN-treated group ($p < 0.05$) (Fig. 7).

### DISCUSSION

The present study conducted experiments using an animal model of acute ligament injury to test the effects of Laennec for sprains or subfailure ligament injuries. Because the number of people engaging in sports and leisure activities is increasing, the number of patients visiting hospitals for various types of sprains is also increasing. Most of the patients are able to achieve favorable outcomes with conservative or nonsurgical treatments. In some cases, however, patients may experience joint instability and chronic pain. While various methods have been attempted to regenerate ligaments in patients with sprains who have undergone unsuccessful conservative treatments, there is not enough clinical evidence.

Our results demonstrated that Laennec has immune suppressive and regenerative effects in damaged ligament. In this study, we did not test anti-inflammation markers such as cyclooxygenase 1 (COX1) and COX2. However, we concluded that Laennec has effects on anti-inflammation with immune suppressive effects based on our histologic analysis which allows to evaluate cellularity, cell shape, and alignment. In other recent studies, the anti-inflammatory effects of Laennec were proven. Furthermore, Laennec is rich in bioactive substances; therefore, we thought that some substances have an anti-inflammatory effect. The inflammatory response is triggered by inflammatory cells that have aggregated around the injured ligament when an acute ligament injury occurs. It causes swelling and pain, which become the main symptoms of patients with sprains. When an injured ligament was treated with saline, dextrose, or PDRN for 4 weeks, the percentage of round cells (inflammatory cells) was high (Fig. 3). However, the group treated with Laennec showed a relatively low percentage of round cells (inflammatory cells) and high percentage of spindle cells (fibrocytes) (Fig. 3). Despite these results, our results confirmed that Laennec effectively promotes the

### Table 2. Semiquantitative Analysis of the Degree of Ligament Injury Using the Modified Bonar Scale

| Group               | Bonar scale (mean ± S.D.) | $p$ Value (vs. normal) |
|---------------------|---------------------------|------------------------|
| Normal              | 4.0 ± 1.7                 |                        |
| Saline at 4 weeks   | 6.7 ± 2.1                 | 0.034                  |
| Laennec at 4 weeks  | 5.2 ± 1.5                 | 0.229                  |
| PDRN at 4 weeks     | 5.5 ± 1.4                 | 0.121                  |
| Dextrose at 4 weeks | 6.5 ± 1.5                 | 0.022                  |
proliferation of fibrocytes for ligament regeneration as described in the previous study by Reeves. Based on such findings, administering Laennec to patients with sprains is expected to quickly reduce inflammation and pain and to promote rapid regeneration of ligaments through the proliferation of fibrocytes. Therefore, future studies are needed to find the active ingredient in Laennec.

The number of cells measured by DAPI staining indicates that the groups treated with saline, dextrose, or PDRN after injury had more cells after 4 weeks, whereas the group treated with Laennec had a similar number of cells as the normal ligament group after 4 weeks. In addition, measurements of activated macrophages by CD68 (ED1) immunostaining show that the groups treated with saline, dextrose, or PDRN after injury had increased macrophage density after 4 weeks, whereas the group treated with Laennec had lower density of macrophage than that of the groups treated with saline, PDRN, or dextrose but similar to that of the normal ligament group.

An injured ligament may become physically weaker and stiffer during the healing process as rigidity increases and elasticity decreases. The group treated with Laennec maintained physical characteristics that were similar to those of the normal ligament group at 1 and 4 weeks after treatment. Since our biomechanical test results show no significant differences in physical characteristics between normal and saline treatment group, more supporting experiments may be necessary. Furthermore, the group treated with PDRN showed a large increase in the tensile modulus compared to that during the first week; therefore, the elasticity of the ligament decreased and the ligament became stiffer at the first week, however the elasticity was recovered at 4 weeks.

In relation to the aforementioned semi-quantitative assess-
Fig. 5. Image of CD68 (ED1) Staining (a); The Calculated Fluorescence Density Is Shown (b)
Kruskal–Wallis test and LSD posttest. *p < 0.05 and **p < 0.01 vs. normal. ##p < 0.01 vs. saline.

Fig. 6. Mechanical Properties after Ultimate Tensile Strength (a) and Modulus (b)
Kruskal–Wallis test and LSD posttest. **p < 0.01 vs. normal.
ment using the Bonar scale, both Laennec and PDRN are effective for ligament regeneration. Although Laennec was able to regenerate the properties of the ligament to a certain degree, PDRN could not regenerate ligament elasticity. When normal fibrocytes were extracted, cultured, and treated with the test drugs in in vitro settings, Laennec significantly facilitated the expression of collagen type I, Sx, and Tnmd. Collagen type I has higher elasticity than type II, and the RT-PCR results indicated that Laennec promoted regeneration of ligaments that were more similar to normal ligaments. However, caution should be taken when interpreting the results of the present study because of its limitations of being an animal study and having a small number of cases and a short study period of just 4 weeks. Therefore, future studies are needed to supplement these results. In addition, the lack of Western blotting or immunohistochemistry at the molecular biology level was a limitation of this study. Furthermore, the histologic analysis (cellularity, shape, and alignment) results may have included bias.

To address this issue, three independent researchers scored the findings separately, and the scores were derived after reaching a consensus regarding any discrepancies in scoring.

CONCLUSION

Laennec did not cause changes in the physical characteristics of the injured ligament; however, it reduced the inflammatory response by immune suppressive effect. Such an anti-inflammatory effect is expected to have the clinical effects of relieving swelling and pain that accompany inflammation and, at the same time, of promoting tissue regeneration. Therefore, our results demonstrate that Laennec promoted regeneration of ligaments that were more similar to normal ligaments. However, caution should be taken when interpreting the results of the present study because of its limitations of being an animal study and having a small number of cases and a short study period of just 4 weeks. Therefore, future studies are needed to supplement these results. In addition, the lack of Western blotting or immunohistochemistry at the molecular biology level was a limitation of this study. Furthermore, the histologic analysis (cellularity, shape, and alignment) results may have included bias.

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