Master of Science

Effect of Polydeoxyribonucleotide on Tendon Regeneration in Tendinopathy Animal Model

힘줄병증 동물모델에서 Polydeoxyribonucleotide 가 힘줄재생에 미치는 영향

The Graduate School

Of the University of Ulsan

Department of Medicine

Hwa-Sun Yang

Effect of Polydeoxyribonucleotide on Tendon Regeneration in Tendinopathy Animal Model

Supervisor Kyounghyo Choi

Master's thesis

Submitted to
The graduate School of the University of Ulsan
In partial Fulfillment of the Requirements
For the Degree of

Master of Science

by

Hwa-Sun Yang

Department of Medicine
Ulsan, Korea
February 2018

Effect of Polydeoxyribonucleotide on Tendon Regeneration in Tendinopathy Animal Model

This certifies that the master's thesis of Hwa-Sun Yang is approved.

심사위원장	김 원	교수님	(인)
심사위원	최경효	교수님	(인)
심사위원	정제훈	교수님	(인)

Department of Medicine

Ulsan, Korea

February 2018

Abstract

Polydeoxyribonucleotide (PDRN) is a mixture of deoxyribonucleotide polymers of different lengths and binds to the A2A receptor. PDRN has been used for the treatments of regeneration. The aim of this study was to investigate the effect of PDRN on tendon regeneration in Achilles tendinopathy.

Fourty-eight Sprague-Dawley rats were used for the study. I injected type I collagenase to rat Achilles tendon to make a tendinopathy model. Four days later, I injected saline for control group, PDRN for experimental group to Achilles tendon. Following the plan, I sacrificed rats at 2 weeks or 4 weeks after the treatment and measured quantitative real-time polymerase chain reaction (qRT-PCR), western blot, histological analysis and mechanical testing.

The maximal stress of 4 week PDRN group was higher than control group (p = 0.026) and the cross-sectional area decreased in the PDRN group at 4 weeks (p = 0.009). The mRNA level of collagen I, collagen III, Tenascin C, and VEGF showed no significant difference in PDRN group at both weeks. Protein level of Tenascin C significantly decreased in 4 weeks PDRN group (p = 0.009), but the protein levels of the rest tendon markers showed no significant differences.

I expect the therapeutic effects of the PDRN and the maximal stress of PDRN group increased. Even though molecular biological evidences for regeneration were not enough in this study, elevated tension ability of PDRN seems to be good treatment to try. The molecular biological evidences might not be detected because the extreme inflammation phase was undergone. To clarify the mechanism of increased maximal stress and decreased cross-sectional area, further studies including the inflammatory markers should be done.

Keywords: Polydeoxyribonucleotide (PDRN), Achilles tendon, Tendinopathy

Contents

List of Figures

Introduction

Tendon is vulnerable to injury because of the limited blood supply and the high tensions on it. Tendinopathy is a chronic degenerative condition that diverse changes also occur to the extracellular matrix, resulting in disorganization of collagen fibers, resulting in the tendons losing their unique biomechanical characteristics used to deliver force energy[1].

Tendinopathy frequently does not respond to available treatments such as rest, training modification, physiotherapy, non-steroidal anti-inflammatory drugs, and corticosteroid injections. Tendinopathy is a common disease and might lead to disability. About 3-10% of chronic tendinopathy may progress to tendon ruptures.[2] However, its therapeutic advancements are limited.

Lots of studies are ongoing to find novel therapies for tendinopathy. Regenerative medicine research seeks to restore tendon function using growth factors, gene therapy, and tissue engineering techniques[3]. Some researchers have brought the light on the importance of the role of the extracellular nucleotides and nucleosides to enhance cell activity and proliferation. Purine nucleotides and nucleosides act as mitogens for fibroblasts, endothelial cells and other cell types[4,5] and interact with other growth factors such as fibroblast growth factor, epithermal growth factor, and platelet-derived growth factor[6,7].

Polydeoxyribonucleotide (PDRN) is the active DNA fraction extracted from trout spermatozoa[8]. PDRN acts through stimulation of the adenosine A2A receptor and is able to induce vascular endothelial growth factor (VEGF) production during pathologic conditions[9] with no toxic of adverse effect[10]. PDRN leads to improvement in angiogenesis in burn wound treatment activating the salvage pathway of nucleic acids to provide available nucleosides and nucleotides[11]. PDRN is commonly used by plastic surgeons in pre-surgical cutaneous treatments, as it stimulates fibroblast metabolism, promoting both an increase in the number of fibroblasts and dermal matrix component production[12]. The effect of PDRN has been analysed in a number

of tissues, such as human corneal epithelium[13], showing improvement of tissue regeneration in all cases.

In light of these backgrounds, I hypothesized that PDRN treatment could be regenerate the damaged Achilles tendon with reducing inflammation. I made rat tendinopathy model by injecting type I collagenase in Achilles tendon and used PDRN for regeneration.

Using genetic tools, I compared the key transcription factors characterized for tendon such as Tenascin C, Type I collagen, Type III collagen, and VEGF for angiogenesis. Also mechanical testing was conducted for examination of tensile stress.

Materials and Methods

Polydeoxyribonucleotide (PDRN)

The concentration of PDRN was 16mg/ml. PDRN was supported by Pharma Research Products Co., Ltd.

Animal Study Design

Fourty-eight 8-week old Sprague-Dawley rats were anesthetized by isofluorane inhalation, respectively. A small incision was made in the skin to expose the Achilles tendon followed by injection of type I collagenase reagent (Thermo Scientific, Waltham, MA) for injury. Rat Achilles tendon has two separated strips with screw pattern so we injected 62.5 UI of collagenase each strip. Four days after the injury, therats were divided into four groups: the control group for two weeks (saline injected around Achilles tendon and sacrificed two weeks after the last injection), the PDRN group for two weeks (PDRN injected and sacrificed two weeks after the last injection), the control group for four weeks (saline injected and sacrificed four weeks after the last injection), the PDRN group for four weeks (PDRN injected and sacrificed four weeks after the last injection). I injected 100 μl of saline/PDRN three times every two days. The rats were euthanized with carbon dioxide for harvesting tendon (Figure 1).

RNA isolation, reverse transcription, and qRT-PCR.

Total RNAs were extracted from Achilles tendons using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RevertAid First strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA) was used to synthesis cDNA from RNA and PCR was performed in a BIO-RAD T100™ - Thermal Cycler (Bio-Rad, Hercules, CA).

qRT-PCR was performed using a LightCycler™ 480 SYBR Green I-Step Kit and the LightCycler™ 480 Instrument II Real Time PCR system (Roche Applied Science,

Mannheim, Germany) according to the manufacturer's instructions. Gene expression was normalized to that of GAPDH, which was used as an internal control. The sequences of the primers used were as follows: Rattus GAPDH, 5'-TCA CTC TAC CCA CGG CAA GTT CAA-3' (sense) and 5'-ACG ACA TAC TCA GCA CCA GCA TCA-3' (antisense); Rattus Collagen I, 5'-CAT CGG TGG TAC TAA C-3'(sense) and 5'- CTG GAT CAT ATT GCA CA -3'(antisense); Rattus Collagen III, 5'-TGC CTA CAT GGA TCA GGC CAA TG-3'(sense) and 5'-TGC TCC ATT CAC CAG TGT GTT TAG-3'(antisense); Rattus Tenascin C, 5'-CAG AAG CTG AAC CGG AAG TTG-3'(sense) and 5'-GGC TGT TGT TGC TAT GGC ACT-3'(antisense); Rattus VEGF, 5'-GAG CAG AAA GCC CAT GAA GTG-3'(sense) and 5'-GGT CTC AAT TGG ACG GCA AT-3'(antisense).

Western Blotting

To examine the production of proteins, isolated Achilles tendons were washed with PBS and dissolved in lysis buffer (Intron, Seoul). To determine levels of tendon regeneration related proteins, tendon tissues were homogenized. Total protein concentrations of the supernatant were measured using a BCA protein assay kit (Thermo Scientific, Waltham, MA). The measured samples were boiled in 5× SDS sample buffer, and all tissue lysates were equally resolved on 8-12% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked using 5% skim milk solution in Tris-buffered saline (20mM Tris/HCl, pH7.6, 150mM NaCl, and 0.1% Tween20) for 1 hour and incubated overnight with the primary antibodies at 4℃. The membrane incubated for 1 hour with the HRP-conjugated secondary antibodies at room temperature and then the immunoreactivity was detected using as enhanced chemiluminescent solution (Thermo Scientific, Waltham, MA). B-actin (1:1000, Abcam, cat: ab, US), Collagen I (1:1000, Abcam, cat: ab34710, US) and Collagen III (1:1000, Abcam, cat: ab7778, US), Tenascin C (1:1000, Abcam, cat: ab108930, US) and VEGF (1:1000, Abcam, cat: ab46154, US).

Hematoxylin-Eosin and Alcian Blue Staining

Isolated rat Achilles tendons were paraffin embedded, and sectioned into 4um. The sections were stained with hematoxylin-eosin (H&E) and Alcian blue. The specimens were examined by standard light microscopy.

Bonar Score

The Bonar score was designed for the tendinopathy assessment[14]. To determine the area of pathological change, regression with post hoc pair-wise analysis was undertaken on the Bonar scores.

Biomechanical Test

After the rats were euthanized with carbon dioxide, entire Achilles tendon was harvested. The tendon was loaded until it ruptured. The load-to-failure with a preload of 0.1N at a rate of 1 mm/s were measured by using a mechanical testing machine (JSV-H1000, JISC, JAPAN) (figure 2A). The custom clamping system consists of 2 different fixtures with a tendon fixation unit and a foot fixation unit (figure 2B). The testing data were automatically collected by a computer-based data acquisition system.

Ethics statement

All animals were reared and treated in strict accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals. All procedures performed in the study were obtained from Animal Review Committee (IACUC of Asan Medical Center Laboratory of Animal Research). Male Sprague-Dawley (8 weeks of age, 300-350g of body weight, n=48) were observed for 7 days in the animal care laboratory before experimental procedures.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using the Mann-Whitney U test for comparing groups. A value of p less than 0.05 was considered significant.

Results

Mechanical evaluation

The cross-sectional area of the tendons was 12.19 ± 4.37 mm² for control group and 15.09 ± 4.34 mm² for PDRN group at 2 weeks, and 11.26 ± 3.83 mm² for control group, 7.78 ± 4.23 mm² for PDRN group 4 weeks, respectively. The cross-sectional area of PDRN group was no significant difference at 2 weeks and significantly decreased at 4 weeks (p = 0.002) (fig. 2C). Mechanical testing of four groups at 2 weeks and 4 weeks showed no significant difference in the maximal load failure between the control group and PDRN group (data not shown). Also no significant difference in the maximal stress was observed in PDRN group at 2 weeks. However, the maximal stress of the PDRN group at 4 weeks was significantly increased (p = 0.026) (fig. 2D).

Histological findings

Rat Achilles tendons were paraffin embedded, and sectioned into 4um. All sections were Hematoxylin and eosin (H&E) and Alcian blue stained for Bonar score. Bonar score is a scoring system for tendon. The higher grades were scored for the more chondroitic changes shown and the more loss of collagen fiber architecture shown. The score was 10.83 ± 0.75 for control group and 11.83 ± 0.75 for PDRN group at 2 weeks, 9.5 ± 1.38 for control group and 9.67 ± 1.63 for PDRN group at 4 weeks. Compared to the control group, the bonar score of PDRN group at 2 and 4 weeks showed an increasing pattern, however, it was not statistically significant ($p > 0.05$) (fig. 3A). Normal tendon collagen is tightly arranged and a few distinct cells are between collagen. Collagen loses its normal polarization pattern such as the saline group when the tendon become pathologically. The collagen arrangement of PDRN group is restored arrangement compared to that of saline group at both weeks (fig. 3B).

mRNA expressions of tendon specific markers

To investigate the growth factors produced in the Achilles tendon, I examined collagen I, collagen III, Tenascin C, and VEGF expression with qRT-PCR. Expression level of collagen I, collagen III, Tenascin C in PDRN group at 2 weeks was slightly increased compared to that of control group but there was no statistically significant. The mRNA level of collagen I, collagen III, Tenascin C of PDRN group at 4 week was slightly decreased but with no statistically significant. No difference was observed also in the VEGF mRNA level of PDRN group compared to control group (fig. 4).

Protein expressions of tendon specific markers

To measure the relative abundance of proteins and to assess the effect of PDRN treatment, proteins were extracted from every tendon sample. I examined the protein of collagen I, collagen III, Tenascin C, and VEGF. In PDRN group of 2 weeks, all protein expression levels showed increasing pattern but there was no significant difference compared to control group ($p > 0.05$). In PDRN group of 4 weeks, protein expression levels of collagen I, collagen III, and VEGF were increased compared to control group ($p > 0.05$). However, the protein expression level of Tenascin C was significantly decreased ($p = 0.009$) (fig. 5).

Discussion

Tendon is all over the flexor parts like fingers, toes, shoulders, elbows, and knees. Tendon injuries, such as chronic degenerative tendinopathy or acute ruptures, are common pathological problem and notorious for its poor quality and slow rate of healing[15]. The Achilles tendon is the largest and strongest tendon in the body, but one of the most likely to be injured[16]. For tendon injury, surgical treatment may be used to repair or replace. However, the clinical outcome has been unsatisfactory due to limitations including high failure rates and risk of injury recurrence[17,18]. These limitations have spurred the development of tissue engineering strategies to make the innate healing of tendon defects[19].

The tendon healing response divided into the predictable three phases; inflammation phase, proliferation/repair phase, and remodeling phase. In the inflammatory phase, the blood clot forms and inflammatory cells migrate to the wound site. Additionally, the recruitment of chemoattractants begins[17,20]. In the proliferation/repair phase, Fibroblasts are recruited to the wounded area and proliferate. In this early phase of healing, tendon matrix is composed of increased amount of collagen III[21,22]. The remodeling phase begins 1 month after the injury. Collagen fibers and tenocytes start to align in the direction of stress[23,24]. Eventually, fibrous tissue of the wounded area changes mature tendon tissue, this process gets for years[25].

PDRN, polydeoxyribonucleotide, is a 350kDa sized DNA fragment extracted from the spermatozoa of trout[8]. PDRN is deoxyribonucleotide polymers with chain length between 50 and 2000 base pairs[26]. PDRN stimulates the binding of adenosine to A2A receptors leading to increase angiogenesis, differentiation of fibroblasts, and collagen synthesis[27]. PDRN is spotlighted recently since PDRN induces differentiation of natural factors already in our body unlike other medications.

Researches of therapeutic effects of PDRN on many fields are actively ongoing. *Castellini* and colleagues have shown that PDRN did not affect cell viability and increased VEGF secretion in cell level. In the present study, we investigated the

effect of PDRN on Achilles tendinopathy. Our study had some limitations that the small animal number of each group, the short-term follow-up time.

For tendon repair, restoration of mechanical strength is important. We examined mechanical testing on the rat Achilles tendon. The PDRN-treated group at 4 week showed a significantly decreased cross-sectional area (fig. 2C). Also, the significant increase of maximal tensile stress was shown in PDRN group at 4 week (fig. 2D). These findings are suggested that PDRN improves the tensile ability of the Achilles tendon. However, there were no significant differences in 2 week groups (fig. 2D).

Bonar score is designed for the assessment of tendinopathy[28]. All sections were scored using bonar scoring system viewed at 100× total magnification, in order to identify the area to evaluate[14]. Sections get high score if they have; chondrocytes, fiber bundles with complete loss of architecture, hypercellularity, vascular clusters, and abundant mucin[14]. The bonar score showed no significant differences in PDRN groups of 2 week and 4 week (fig. 3A). Histological score was similar between control group and PDRN group. PDRN injection did not effect to histological changes.

Expression level of Collagen I did not show definite change in qPCR (fig. 4A) and western blotting (fig. 5A) after PDRN injections. Compared to control group, mRNA level of collagen III was no difference in 4 week PDRN group (fig. 4B). However, the protein level of collagen III tends to increase without statistically significance (fig. 5B). Collagen I synthesis is a crucial step to determine the mature tendon and the tensile strength[29,30] but no molecular biological evidence found in this study. However, collagen III is the initiator of the tendon healing process and the protein, the final product in our body, level of collagen III in 4 week PDRN group increased. This result goes with the increased maximal stress the increased collagen III supported the tensile ability.

I did not expect this reduction but the protein level of Tenascin C decreased in 4 week PDRN group, unlike the collagen III (fig. 5C). Tenascin C level of tendon showed down-regulated because tendon maturation might not be done. Some studies showed that PDRN increases VEGF and I expected same pattern this study. However,

VEGF expression was no statistically difference after the injection of PDRN (fig. 4D, fig. 5D). It is because the VEGF level of control group increased as much as the PDRN group due to the high regenerative ability of rats.

PDRN has anti-inflammatory effect and regenerative effect[11,18]. These interactions might induce regeneration of tendon not enough. Especially the reduction of cross-sectional area assumes that the anti-inflammation was going on PDRN group at 4 weeks.

In this study, I tried to reveal the therapeutic benefits of PDRN on tendon healing. At last, I found that PDRN injection enhances the tensile stress and downsizes cross-sectional area with increased collagen III of the Achilles tendon at 4 week. However, the molecular biological evidence was scarce. These results come out because the anti-inflammatory effect or PDRN might have little therapeutic effect. Further studies should be carried out to reveal the mechanism of PDRN in healing phase with long-term follow-up and to find more certain evidences for application in humans.

국문요약

힘줄병증은 과도한 사용과 노화로 인한 힘줄의 퇴행성 질환이며, 매우 흔한 근골격계 질환군으로 인구가 노령화 되어감에 따라 힘줄병증 환자가 증가하는 추세이다. 힘줄 조직은 혈관분포가 적고 재생 능력이 낮기 때문에 수술이나 치료의 한계가 있다.

PDRN 은 polydeoxyribonucleotide 라는 DNA 조각으로서 체내에서 아데노신 2 수용체에 작용하여 항염증 반응과 조직 재생을 활성화한다. 현재 화상 치료에도 사용되며 최근 근골격계 질환에서도 일부 사용하지만, 명확한 기초 실험으로 입증되지 못해 본 연구에서는 이런 임상 결과를 뒷받침할 기초연구를 진행하였다.

우리는 힘줄병증 모델을 만들기 위해 흰쥐의 아킬레스건에 콜라제네이즈를 주입하여 염증을 유도하였고, 이 후 4 일이 지난 시점부터 대조군에는 생리식염수를, 실험군에는 PDRN 을 2 일 간격으로 각각 3 번씩 주입했다. 마지막 주입 후 각 군의 아킬레스건을 2 주, 4 주 간격으로 채취하였다.

힘줄병증의 재생 정도를 확인하기 위해 힘줄의 두께 측정과 인장력 시험 및 면역 염색을 시행하였고, 힘줄 표지자인 CollagenⅠ, CollagenⅢ, Tenascin C 와 혈관 분포의 증감을 확인할 VEGF 에 대한 qRT-PCR 과 western blot 을 실시하였다.

그 결과, 조직학적 소견에는 2, 4 주차 모두 대조군과 실험군의 유의한 차이가 없었다. 최대 스트레스는 4 주차 실험군에서 유의하게 증가했고, 단면적도 감소하였다 (p = 0.026, 0.009). mRNA 는 2, 4 주차 모두 유의한 차이가 없었다. 단백질 수준은 4 주차 실험군에서만 Tenascin C 가 유의하게 감소하였다 (p = 0.009).

우리는 이 연구에서 콜라제네이즈-유도 힘줄병증 모델에서 PDRN 의 치료 효과를 기대하였지만, 최대 스트레스가 증가하고 단면적이 감소하는 것 외에 다른 재생을 증가시키는 근거를 찾지 못했다.

PDRN 의 주입으로 4 주차 실험군의 힘줄 장력이 좋아지는 결과를 확인했다. 실험군에서의 최대 장력 증가와 단면적이 감소하는 기전을 확인하기 위해 항염증 지표를 포함한 추가적인 실험이 필요하다.

중심단어: PDRN, 아킬레스건, 힘줄병증

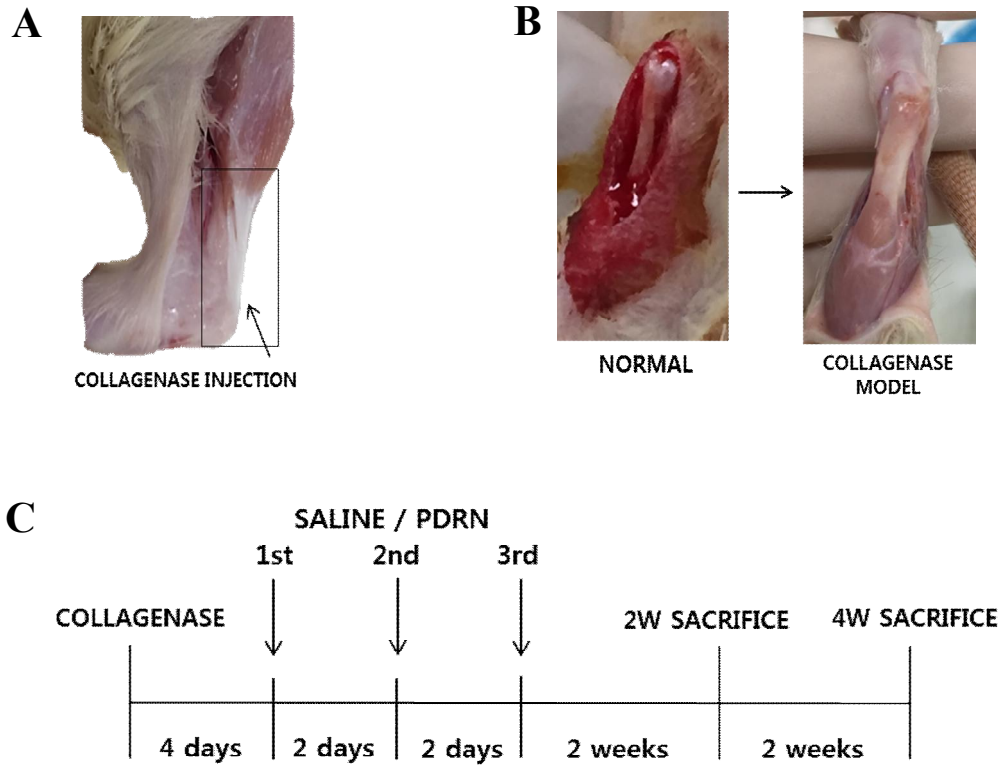

Figure 1. Animal study design

Injection point was 3mm from the heel of the rat (A) and the collagenase model of rat Achilles tendon (B). We injected saline for control group and PDRN for experimental group three times every two days (C).

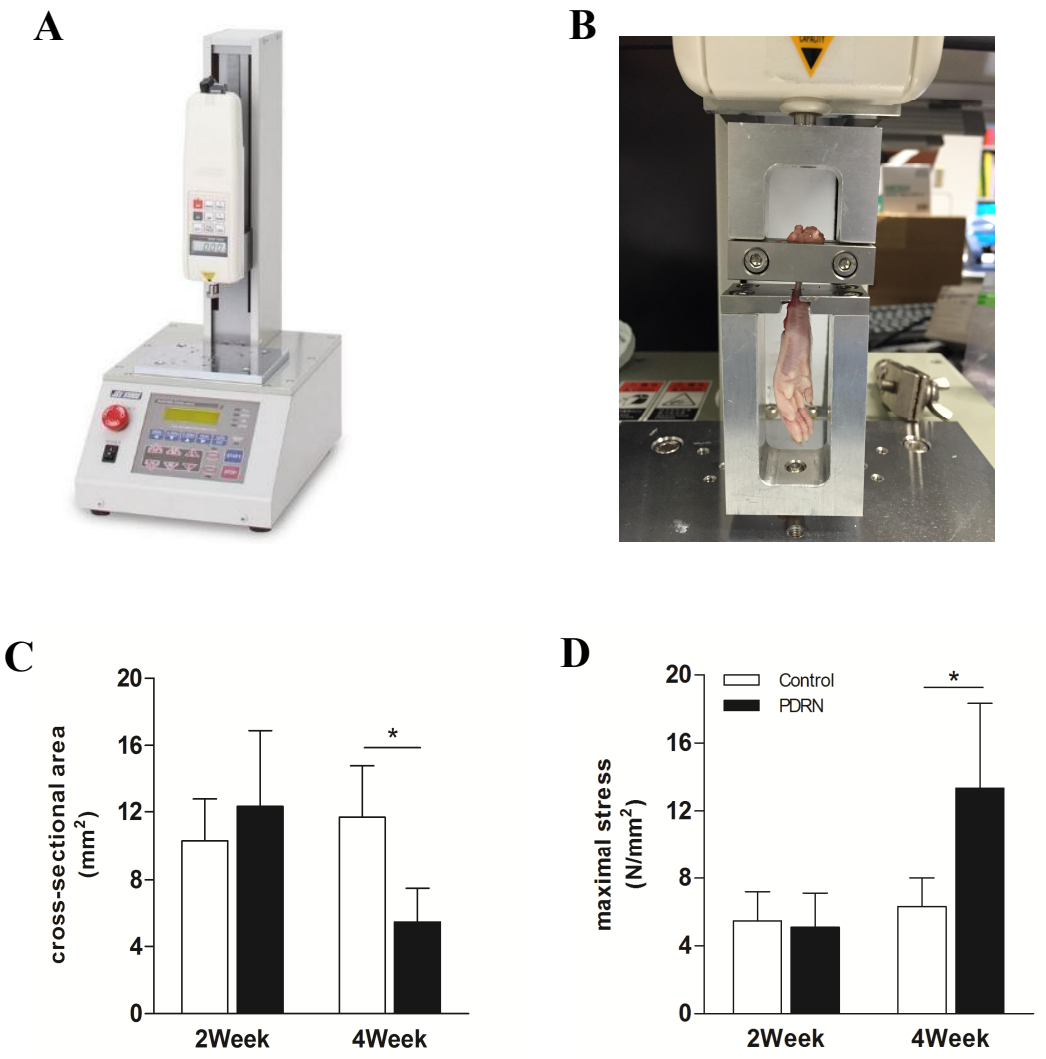

Figure 2. Mechanical testing of Achilles tendons

Maximal stress was examined using JSV-H100 mechanical testing machine (A) and the Achilles tendon of rat was fixed the clamps (B). Cross-sectional area was calculated by measured thickness (C) and load-to-failure divided by cross-sectional area (D). *$p < 0.05$.

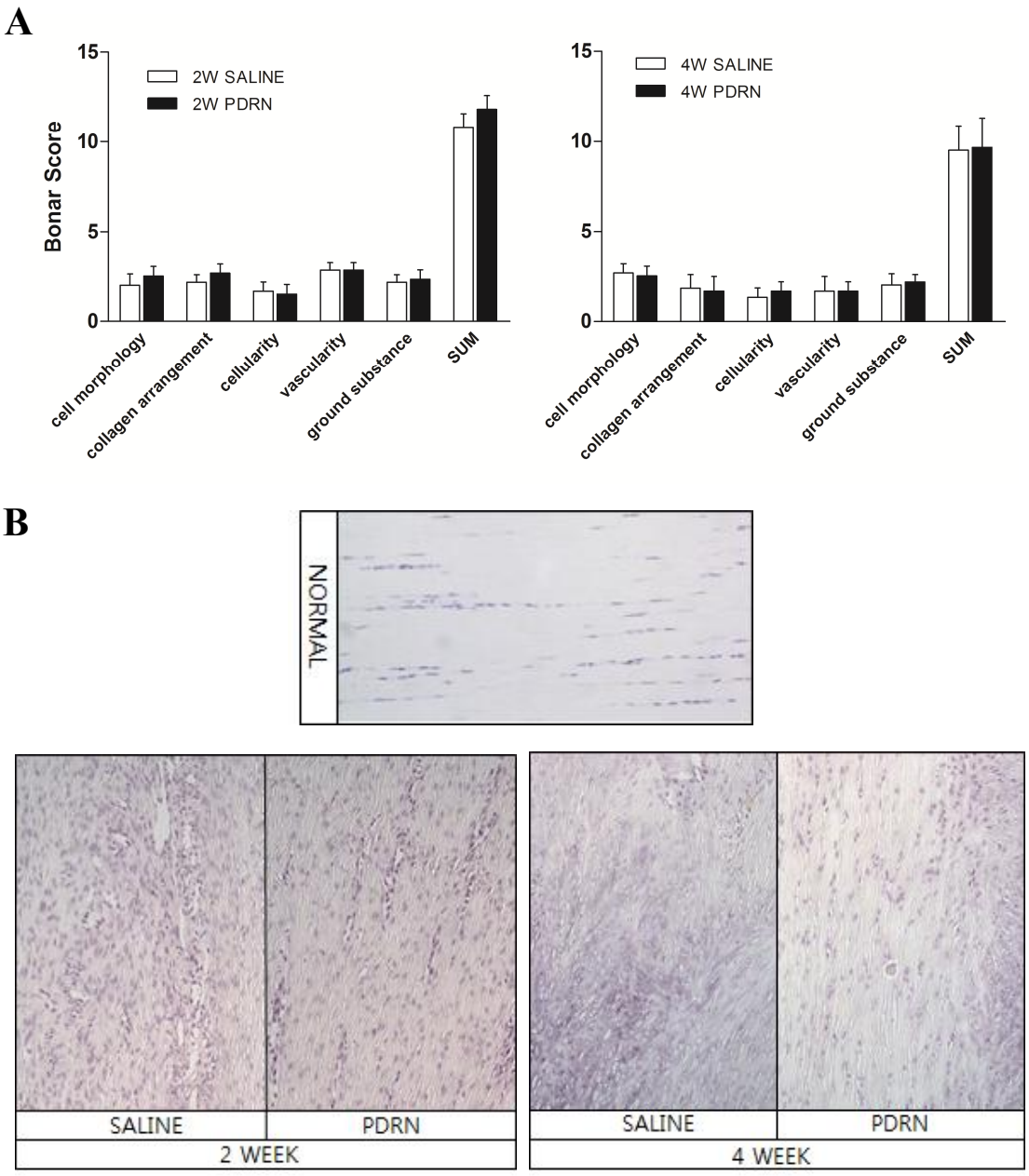

Figure 3. Histological findings

The sections were stained with hematoxylin-eosin (H&E) and Alcian blue. All slides were scored using Bonar scoring system under a light microscopy (A). Collagen arrangements of normal Achilles tendon, control group, and PDRN group (B).

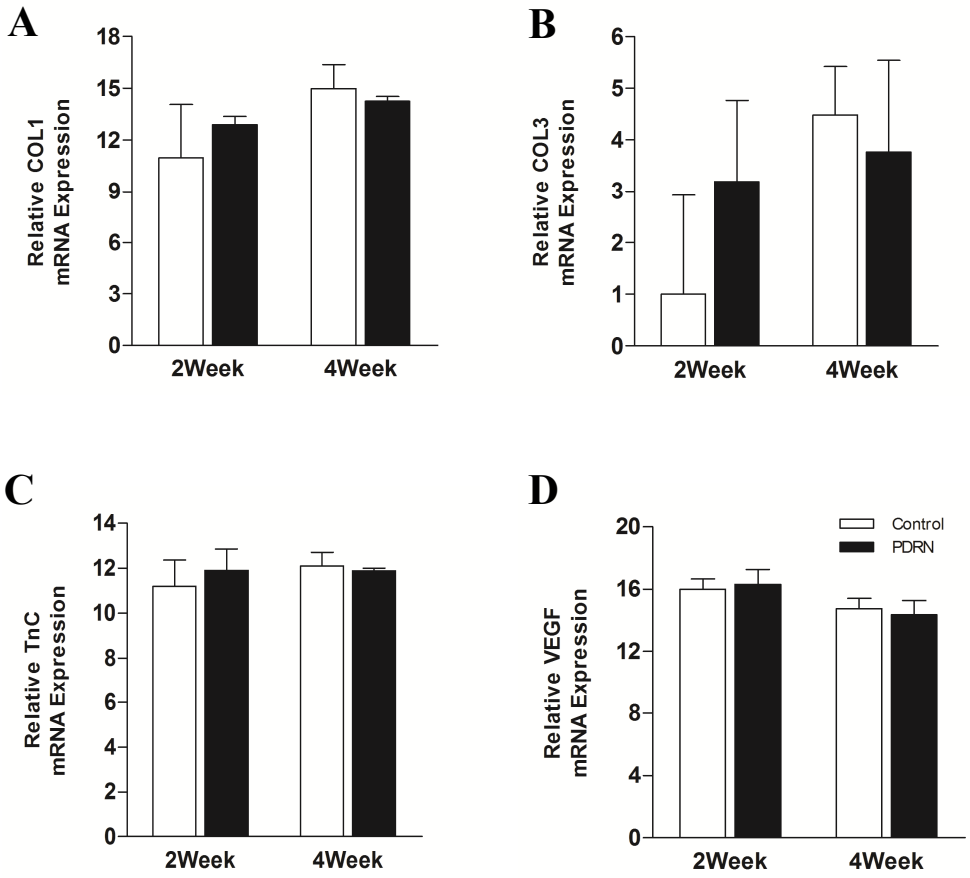

Figure 4. mRNA levels of tendon markers

All Achilles tendon samples were assayed by quantitative real-time PCR (qPCR) using collagen1, collagen3, TenascinC, or VEGF-specific primers. All representative data are mean ± s.d.. *$p < 0.05$.

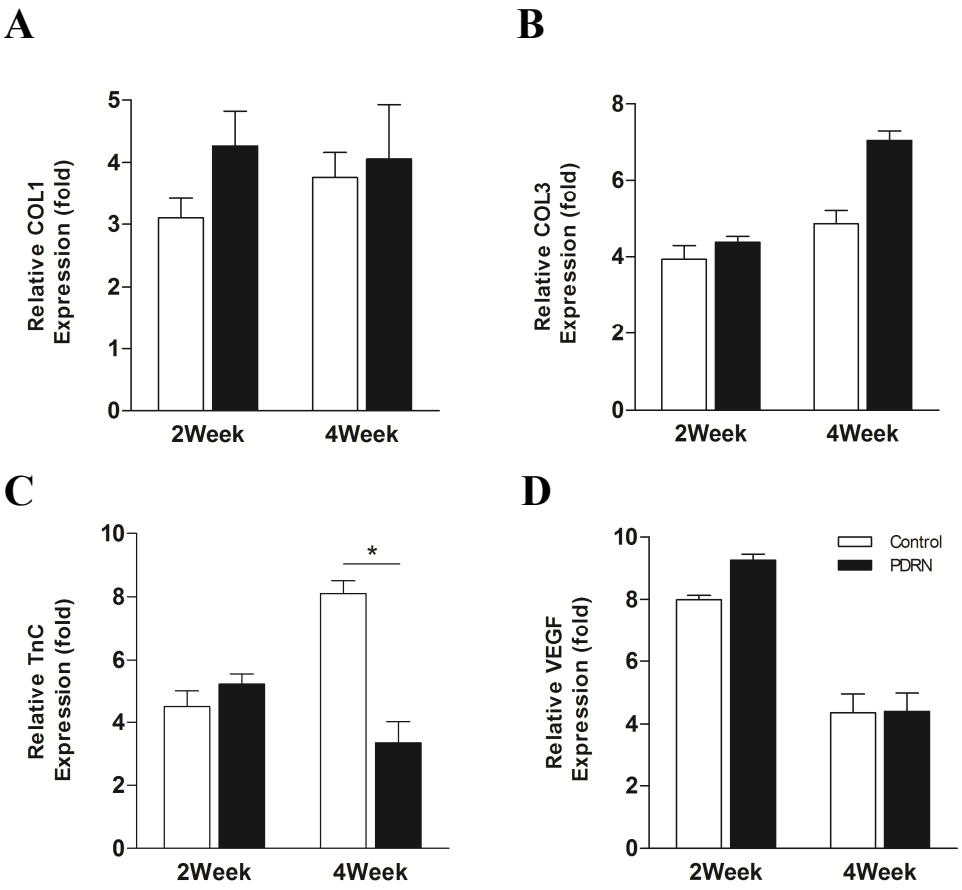

Figure 5. Protein levels of tendon markers

All tendon samples were measured by western blot using Collagen I, Collagen III, Tenascin C, or VEGF-specific antibodies. The Collagen III level was upregulated in the 4 week PDRN group, but the value of p was over 0.05 (B). The Tenascin C level of PDRN group at 4 week was significantly decreased (C). *$p < 0.05$.

References

1 Wang, J. H. Mechanobiology of tendon. *J Biomech* **39**, 1563-1582, doi:10.1016/j.jbiomech.2005.05.011 (2006).

2 Kvist, M. Achilles tendon injuries in athletes. *Ann Chir Gynaecol* **80**, 188-201 (1991).

3 Sharma, P. & Maffulli, N. Tendon injury and tendinopathy: healing and repair. *J Bone Joint Surg Am* **87**, 187-202, doi:10.2106/JBJS.D.01850 (2005).

4 Rathbone, M. P. *et al.*Purine nucleosides and nucleotides stimulate proliferation of a wide range of cell types. *In Vitro Cell Dev Biol* **28A**, 529-536 (1992).

5 Thellung, S., Florio, T., Maragliano, A., Cattarini, G. & Schettini, G. Polydeoxyribonucleotides enhance the proliferation of human skin fibroblasts: involvement of A2 purinergic receptor subtypes. *Life Sci* **64**, 1661-1674 (1999).

6 Wang, D. J., Huang, N. N. & Heppel, L. A. Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. *Biochem Biophys Res Commun* **166**, 251-258 (1990).

7 Chavan, A. J. *et al.*Interaction of nucleotides with acidic fibroblast growth factor (FGF-1). *Biochemistry* **33**, 7193-7202 (1994).

8 Altavilla, D. *et al.*Activation of adenosine A2A receptors restores the altered cell-cycle machinery during impaired wound healing in genetically diabetic mice. *Surgery* **149**, 253-261, doi:10.1016/j.surg.2010.04.024 (2011).

9 Altavilla, D. *et al.*Polydeoxyribonucleotide (PDRN): a safe approach to induce therapeutic angiogenesis in peripheral artery occlusive disease and in diabetic foot ulcers. *Cardiovasc Hematol Agents Med Chem* **7**, 313-321 (2009).

10 Galeano, M. *et al.*Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Wound Repair Regen* **16**,208-217, doi:10.1111/j.1524-475X.2008.00361.x (2008).

11 Guizzardi, S. *et al.*Polydeoxyribonucleotide (PDRN) promotes human osteoblast proliferation: a new proposal for bone tissue repair. *Life Sci* **73**, 1973-1983 (2003).

12 Bigliardi, E. & Vegni-Talluri, M. Electron microscopic study of interstitial cells in cryptorchid human testes. I: Interstitial cells in prepubertal age. *Andrologia* **14**, 276-283 (1982).

13 Lazzarotto, M., Tomasello, E. M. & Caporossi, A. Clinical evaluation of corneal epithelialization after photorefractive keratectomy in patients treated with polydeoxyribonucleotide (PDRN) eye drops: A randomized, double-blind, placebo-controlled trial. *Eur J Ophthalmol* **14**, 284-289 (2004).

14 Fearon, A., Dahlstrom, J. E., Twin, J., Cook, J. & Scott, A. The Bonar score revisited: region of evaluation significantly influences the standardized assessment of tendon degeneration. *J Sci Med Sport* **17**, 346-350, doi:10.1016/j.jsams.2013.07.008 (2014).

15 Maffulli, N., Longo, U. G., Maffulli, G. D., Khanna, A. & Denaro, V. Achilles tendon ruptures in elite athletes. *Foot Ankle Int* **32**, 9-15, doi:10.3113/FAI.2011.0009 (2011).

16 Calleja, M. & Connell, D. A. The Achilles tendon. *Semin Musculoskelet Radiol* **14**, 307-322, doi:10.1055/s-0030-1254520 (2010).

17 Voleti, P. B., Buckley, M. R. & Soslowsky, L. J. Tendon healing: repair and regeneration. *Annu Rev Biomed Eng* **14**, 47-71, doi:10.1146/annurev-bioeng-071811-150122 (2012).

18 Yang, G., Rothrauff, B. B. & Tuan, R. S. Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. *Birth Defects Res C Embryo Today* **99**, 203-222, doi:10.1002/bdrc.21041 (2013).

19 Kuo, C. K., Marturano, J. E. & Tuan, R. S. Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs. *Sports Med Arthrosc Rehabil Ther Technol* **2**, 20, doi:10.1186/1758-2555-2-20 (2010).

20 Okamoto, N. *et al.*Treating Achilles tendon rupture in rats with bone-marrow-cell transplantation therapy. *J Bone Joint Surg Am* **92**,

2776-2784, doi:10.2106/JBJS.I.01325 (2010).

21 Garner, W. L., McDonald, J. A., Koo, M., Kuhn, C., 3rd & Weeks, P. M. Identification of the collagen-producing cells in healing flexor tendons. *Plast Reconstr Surg* **83**, 875-879 (1989).

22 Juneja, S. C., Schwarz, E. M., O'Keefe, R. J. & Awad, H. A. Cellular and molecular factors in flexor tendon repair and adhesions: a histological and gene expression analysis. *Connect Tissue Res* **54**, 218-226, doi:10.3109/03008207.2013.787418 (2013).

23 Sharma, P. & Maffulli, N. Biology of tendon injury: healing, modeling and remodeling. *J Musculoskelet Neuronal Interact* **6**, 181-190 (2006).

24 Abrahamsson, S. O. Matrix metabolism and healing in the flexor tendon. Experimental studies on rabbit tendon. *Scand J Plast Reconstr Surg Hand Surg Suppl* **23**, 1-51 (1991).

25 Miyashita, H., Ochi, M. & Ikuta, Y. Histological and biomechanical observations of the rabbit patellar tendon after removal of its central one-third. *Arch Orthop Trauma Surg* **116**, 454-462 (1997).

26 Tonello, G. *et al.*Characterization and quantitation of the active polynucleotide fraction (PDRN) from human placenta, a tissue repair stimulating agent. *J Pharm Biomed Anal* **14**, 1555-1560 (1996).

27 Chan, E. S. *et al.*Adenosine A(2A) receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. *Arthritis Res Ther* **15**, R58, doi:10.1186/ar4229 (2013).

28 Maffulli, N., Longo, U. G., Franceschi, F., Rabitti, C. & Denaro, V. Movin and Bonar scores assess the same characteristics of tendon histology. *Clin Orthop Relat Res* **466**, 1605-1611, doi:10.1007/s11999-008-0261-0 (2008).

29 James, R., Kesturu, G., Balian, G. & Chhabra, A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *J Hand Surg Am* **33**, 102-112, doi:10.1016/j.jhsa.2007.09.007 (2008).

30 Kajikawa, Y. *et al.*Platelet-rich plasma enhances the initial mobilization of circulation-derived cells for tendon healing. *J Cell Physiol* **215**, 837-845, doi:10.1002/jcp.21368 (2008).