# Substance P Inhibitor (Neurokinin-1 Antagonist) Promoted Tendon Healing in Collagenase Induced Rat Model of Tendinopathy.

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# Introduction

- Tendinopathy는 사회적으로 문제다.
- 발병율이 높고 잘 안 낫는다.
- 수술까지 해야하는 경우가 있다.
- 발병 원인으로 Substance P(SP)가 제시되고 있다.
- 따라서, SP의 receptor인 neurokinin-1 receptor를 억제하면 좋아질 수 있을 것이다.
- 하지만 지금까지 neurokinin-1 receptor antagnoist의 tendon healing에 대한 effect를 연구한 적은 없다.
- 우리 연구의 목적은 SP inhibitor가 tendon healing에 어떤 영향을 미치는지 확인하는 것이다.
- 이를 위해 우리는 우선 in vitro study로 inflammed tenocyte에 SP inhibitor가 미치는 영향을 분석하고, 이후 in vivo study로 tendinopathy rat에 SP inhibitor treatment 후 회복 정도를 분석하였다.

# Materials and Methods
# 다른 논문에서 참고한 문장들임. 필요시 수정 요함.

- ***Reagents***
- Substance P antagonist (SPA) was obtained from Sigma Chemical (St. Louis, Missouri) for use in cell culture. Dulbecco modified Eagle medium, penicillin-streptomycin, and fetal bovine serum were supplied by Gibco Laboratories (Grand Island, New York).

# Materials and Methods

- ***Isolation and culture of tenocyte***
- In vitro experiments: cell preparation. Human Tenocytes were purchased (Zenbio, NC, USA) and cultured with DMEM and incubated in 5% CO2, 95% air at 37°C. The culture medium was renewed twice a week. When cells became confluent, the tendon fibroblasts were trypsinized with 0.25% trypsin (weight per volume)-EDTA solution and subcultured onto 100 mm cell culture dishes at a seeding density of $1 \times 10^6$ cells per flask under the same culture conditions. The culture medium was renewed twice a week. Cells from the second, third, and fourth passages were used in the following experiments.

# Materials and Methods

- **Viability test: To determine the dose of NK-1 receptor antagonist, we performed the viability test after adding various doses of NK-1 receptor antagonist**
- A total of 2000 cells were seeded in 100 μl of DMEM in each well of two 96-well plates and were incubated in DMEM with 10−6-M to 10−4-M SPA for seventy-two hours. Control cells were incubated with DMEM only. Cell proliferation was measured by a water soluble tetrazolium salt (WST) assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Then, 10 μl of WST was added to each well and cultures were incubated for an additional two hours at 5% CO2 and 37°C prior to spectrophotometric evaluation. The conversion of WST to formazan was spectrophotometrically measured at 450 nm. Results were normalized and were presented as the percentage of the viable cells in the control group.

# Materials and Methods

- **Validation of SP inhibitor activity: 앞에서 정한 SP inhibitor의 용량이 SP를 적절히 억제하는지 확인하기 위해 수행함.**
- The activity of substance P was determined by western blot analysis. Proteins were separated on SDS-polyacrylamide gels, and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Antibodies specific for PLC, Akt, MAPK and GAPDH were obtained from Cell Signaling Technology (Beverly, MA). Proteins were detected with an enhanced chemiluminescence Western blotting kit (Amersham Biosciences, NJ, USA), according to the manufacturer's instructions.

# Materials and Methods

- **in vitro anti-inflammatory effects of SP inhibitor**

The in vitro anti-inflammatory effects of SP inhibitor were tested using tenocytes. In order to demonstrate the in vitro anti-inflammatory effects of SP inhibitor on LPS-treated tenocytes, we analyzed the mRNA levels of COX-2, IL-6, Col Ⅰ and Col Ⅲ using real-time PCR.

Tenocytes ($1 \times 10^6$ cells) were carefully treated with SP inhibitor in 100mm cell culture dishes. After 24 h of incubation, LPS ($10^{-4}$ M) was applied to the tenocytes in all groups. After three days, the cells were harvested. The total RNA was isolated from cells in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA (1$\mu$g) was reverse-transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Amplification of total RNA was performed using the Taq Man Gene Expression Assay (Applied Biosystems, Warrington, UK) for the analyses of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI code: Hs2786624_g1), COX-2 (Hs00153133_m1), IL6 (Hs00174131_m1) COL Ⅰ (Hs00164004_m1) , and COLⅢ (Hs00943809_m1), respectively. PCR amplification and detection were performed using an ABI7300 Real-Time Thermal Cycler (Applied Biosystems, Foster, CA, USA). The relative mRNA levels COX-2, IL-6, Col Ⅰ and Col Ⅲ were normalized to those of glyceraldehyde 3-phophate dehydrogenase (GAPDH).

Also western blot analyses were used to determine the protein level of IL-6, Col Ⅰ and Col Ⅲ. Briefly, Proteins were separated on SDS-polyacrylamide gels, and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Antibodies specific for IL-6 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and those for Col Ⅰ and Col Ⅲ from Cell Signaling Technology (Beverly, MA). Proteins were detected with an enhanced chemiluminescence Western blotting kit (Amersham Biosciences, NJ, USA), according to the manufacturer's instructions.

# Table 1. List of Primers

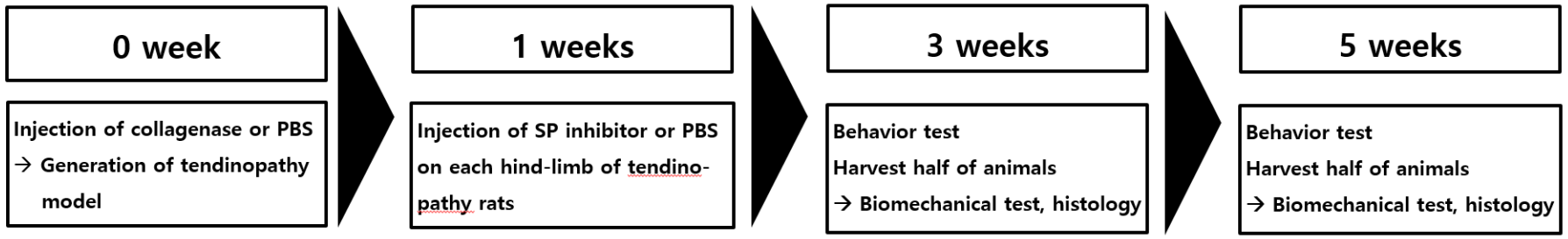
| GENE | Assay ID | Gene Aliases |
|---|---|---|
| GAPDH | Hs02786624_g1 | G3PD, GAPD, HEL-S-162eP |
| PTGS2 | Hs00153133_m1 | COX-2, COX2, GRIPGHS, PGG/HS, PGHS-2, PHS-2, hCox-2 |
| IL-6 | Hs00174131_m1 | BSF-2, BSF2, CDF, HGF, HSF, IFN-beta-2, IFNB2, IL-6 |
| COL I | Hs00164004_m1 | EDSC, OI1, OI2, OI3, OI4 |
| COL Ⅲ | Hs00943809_m1 | EDS4A |

# Materials and Methods

## Animals

- This study was approved and conducted according to regulations and guidelines of the CHA University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used. Male C57Bl/6 mice were obtained from the Orient Bio (Seongnam, Korea) and maintained in the animal facility of CHA University under 12-h light/dark cycle with food and water available ad libitum.

# Fig. 1. *In vivo* study design




1 week있다가 SPI injection 한 이유: **Adipose-Derived Stem Cells Improve Collagenase-Induced Tendinopathy in a Rat Model. (꼭 읽어보자)**

# Table. 2. Animal group

| Group | Treatment | | Site | Number of limb |
|---|---|---|---|---|
| | 0 week | 1 week | | |
| Control | PBS (20 ul) | PBS (20 ul) | Both hindlimb | 8 |
| Tendinopathy | Collagenase (20 ul) | PBS (20 ul) | Rt hindlimb* | 12 |
| SP inhibitor | Collagenase (20 ul) | SP inhibitor (20 ul) | Lt hindlimb* | 12 |

*In the same rat, Rt side was injected by PBS and Lt side was injected by SP inhibitor after animal model was generated.

# Materials and Methods

- **Animal Models of Collagenase-Induced Achilles Tendinitis (다른 논문 것을 가져온 것, 적절히 수정 요함.)**

We established an Achilles tendinitis animal model (using collagenase injection) to demonstrate the in vivo anti-inflammatory and tendon healing effects of SIM/PMSs, as described in our previous study [21]. The in vivo animal experiments were performed and approved by the Institutional Animal Care and Use Committee of the Korea University Medical Center (KOREA-2016-0250, 29 November 2016). SD rats (8-week-old males, DooYeol Biotech, Seoul, Korea) were anesthetized with isoflurane (1% w/v in 2 L oxygen). The rats received a single injection of 50 L of collagenase type I in the right Achilles tendon (Col I) (50 mg/mL inn PBS (pH 7.4)). Seven days after the collagenase injection, 10 mg of the microspheres were mixed with 1 mL of 2% carboxymethyl cellulose (CMC) solution. Randomly selected rats received an injection of 50 L of CMC solution of the microspheres. The real treatment dosages of simvastatin in each SIM/PMS group were 2.39 g/rat for SIM (1 mM)/PMSs and 12.18 g/rat for SIM (5 mM)/PMSs. Simvastatin (105 g/rat) was used as the drug control, and was injected into the Achilles tendon of control animals. Six weeks after treatment with PMSs, simvastatin, SIM (1 mM)/PMSs, and SIM (5 mM)/PMSs, the rats were euthanized for further analysis. The rats were divided into the following six groups: (I) control (no treatment), (II) Col (I) (collagenase treatment), (III) Col (I) + PMSs, (IV) Col (I) + simvastatin, (V) Col (I) + SIM (1 mM)/PMSs, and (VI) Col (I) + SIM (5 mM)/PMSs.

# Materials and Methods

- **Incapacitance test**
- Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury [26]. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value considered for each animal was the mean of 5 consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating a postural equilibrium, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. Data are expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one (Δ Weight). (Ref: Low dose native type II collagen prevents pain in a rat osteoarthritis model)

# Materials and Methods

- **Biomechanical Test**

The tendon specimens were fixed to a specially designed device, which allowed the specimen to be oriented such that a tensile load could be applied along the axis of the tendon. The fixed specimen device was tested on an Instron Mechanical Tester (AG-10KNX, Shimadzu, Kyoto, Japan) at a cross-head speed of 5 mm/min. A 1 newton load cell was used to measure the loading force. The ultimate tensile strength (defined as maximum stress or force per unit area) and stiffness (force required per unit displacement) were also obtained.

아래는 또 다른 예시

- The specimens were thawed. The proximal Achilles tendons were covered with gauze and sutured with nylon (6&66) monofilament yarn (Alfresa Pharma). The tendon and calcaneus were placed in specially designed devices using polymethyl methacrylate resin and placed vertically in a tensile strength sensor (AG-I; Shimadzu) (Figure 1B). Prior to performing the tensile test, the tissues were preconditioned with a static preload of 0.2 N for 1minute, followed by 5 cycles of loading and unloading at a strain amplitude of approximately 0.5% at 60 mm/min. Immediately after preconditioning, the maximum failure load was recorded at a uniaxial tension of 60 mm/min. The maximum failure load was measured as the primary outcome, and the tendon stiffness was calculated from the load-deformation curve (n ¼ 6 per group). This number was backed up by a power analysis (a ¼ 0.05; power level, 80%; SD, 20%; effect size, 0.68).

# Materials and Methods

- **Histopathological Evaluations**
- The calcaneus-Achilles tendon specimens were harvested from the sacrificed rats and fixed in 3.7% (v/v) paraformaldehyde for histological evaluations. The specimens were then dehydrated in ethanol and embedded in paraffin. The tissue blocks were sectioned longitudinally in the rotary microtome (HM 355S automatic microtomes, Thermo Scientific, Waltham, MA, USA) at 5 m thickness. These sliced tissue samples were stained with H & E and Alcian blue.

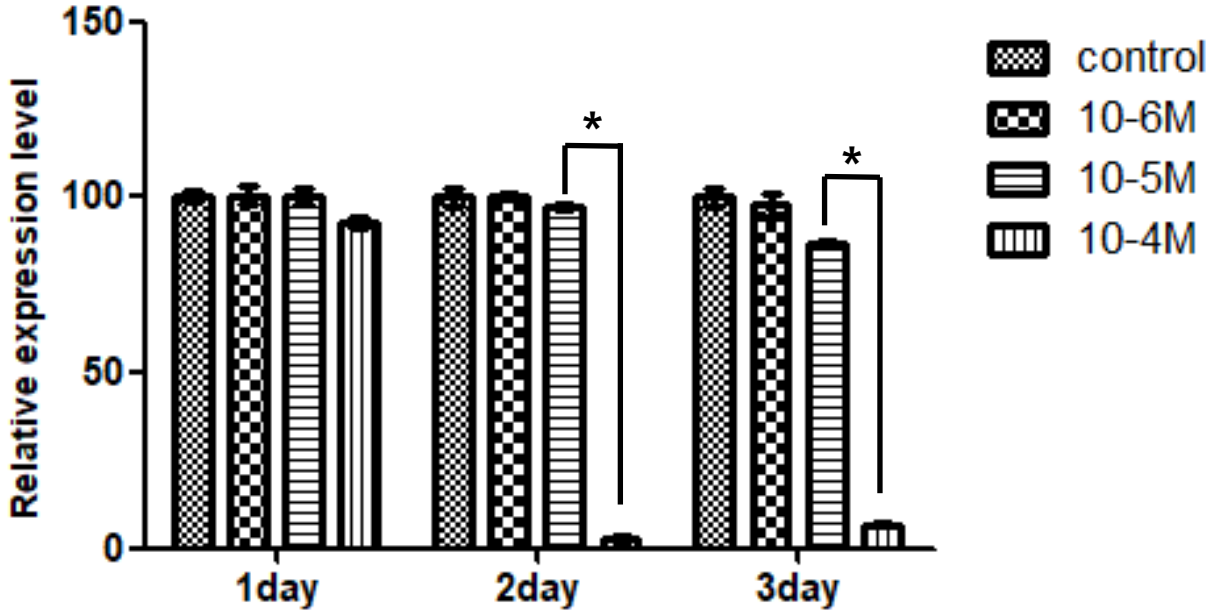
# Materials and Methods

- **Statistical Analysis**

Data are presented as means  standard deviations. Statistical comparisons were carried out via one-way analysis of variance (ANOVA) using Systat software ver. 13 (SigmaPlot, IL, USA). Differences were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$.

# Results

## Fig. 2. Changes of cell viability after SP inhibitor treatment

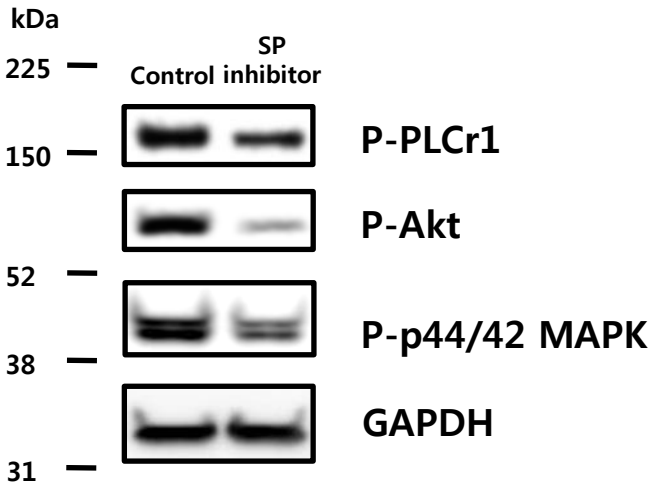


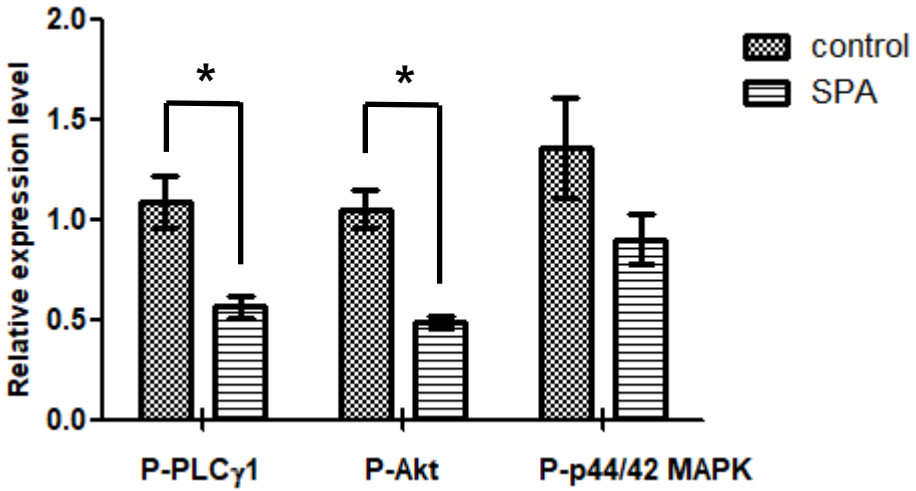

To determine the dose of SP inhibitor, various doses of SP inhibitor was treated in the culture medium during tenocyte culture and the cell viability was analyzed by WST-1 assay. The result showed that the cell viability was decreased significantly at the dose of 10-4 M of SP inhibitor from 48 hours after treatment. However, cell viability was maintained above 80% from the dose of 10-5 M SP inhibitor.

80%를 기준으로 한 이유 ref: HaCaT Keratinocytes Response on Antimicrobial Atelocollagen Substrates: Extent of Cytotoxicity, Cell Viability and Proliferation.

# Results

## Fig. 3. Validation of SP inhibitor activity at 10-5 M

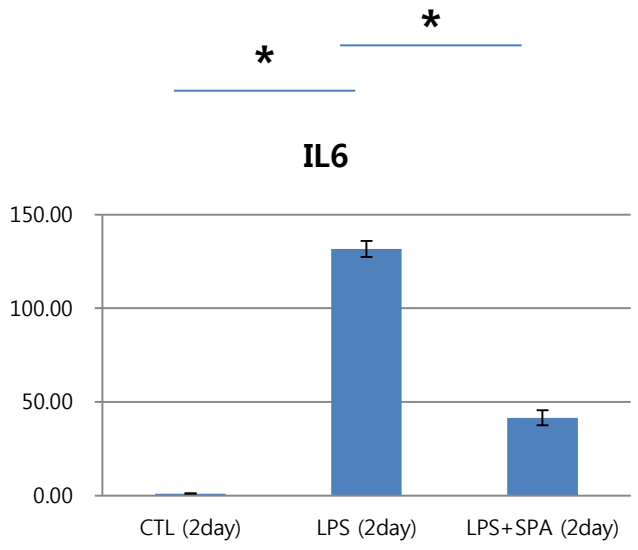


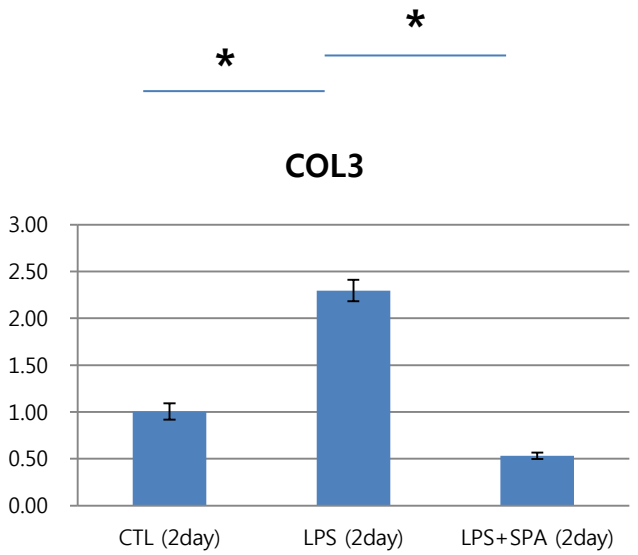



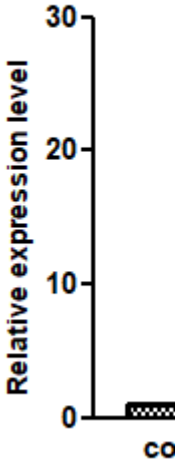

Because it is not clear whether the SP inhibitor could suppress the SP related pathway or not at the dose of 10-5M, the proteins relating to SP pathway including PLC, Akt, MAPK were analyzed after adding 10-5M SP inhibitor in the culture medium during tenocyte culture by western blot. We found that SP inhibitor decreased the major proteins relating to SP pathway including PLC, Akt, MAPK at 10 -5 M.

# Results

- **Fig. 4. Anti-inflammatory effect of SP inhibitor on the inflamed tenocyte**
- To determine the effect of SP inhibitor on the inflamed tenocyte, LPS was used to induce the inflammation on the tenocyte. Proper induction of inflammation on the tenocyte was confirmed by the relevant gene expressions including IL-6 and COX-2.
- Next, SP inhibitor was treated in the inflamed tenocyte. Interestingly, both expression of IL-6 and COX-2 were decreased significantly 2 days after SP inhibitor treatment.



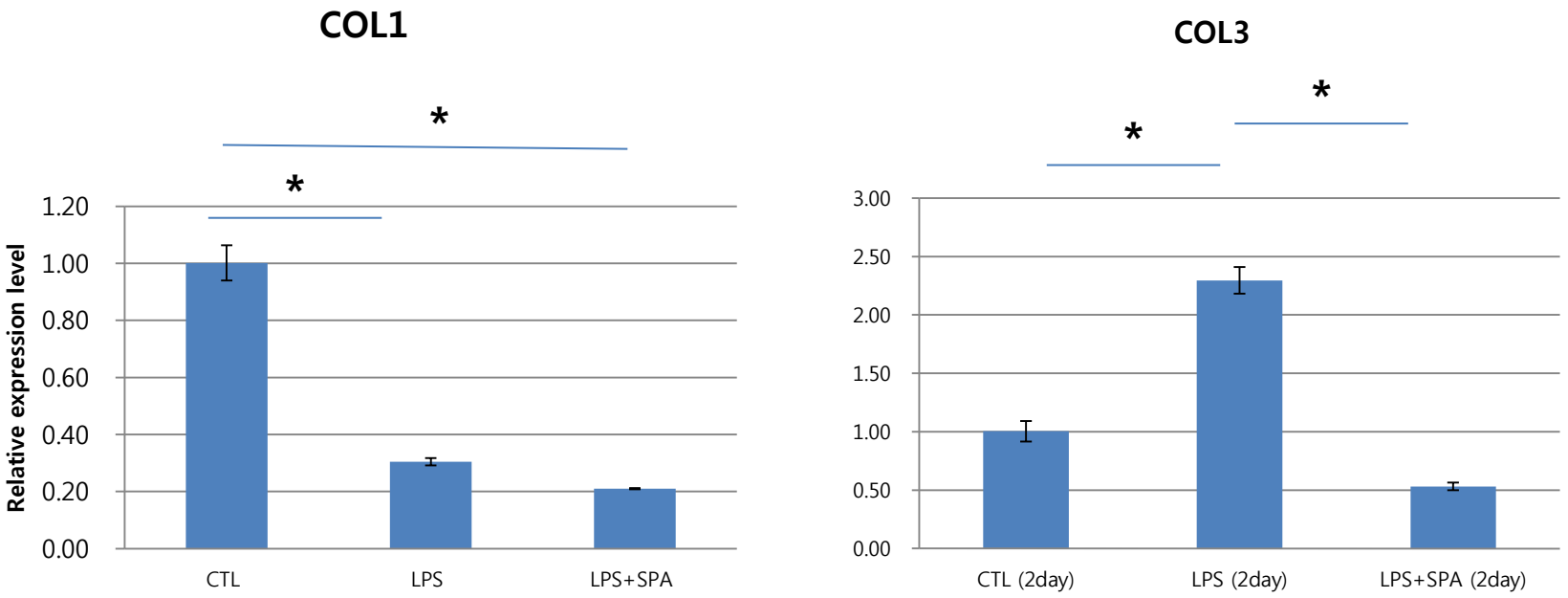



# Results

**Fig. 5. Effect of SP inhibitor on the collagen expression of tenocyte**

There was no significant effect on the expression of type 1 collagen by SPA treatment, but inflamed tenocyte had the higher expression of immature collagen (type 3 collagen). SP inhibitor treatment decreased COL3 in the inflamed tenocyte

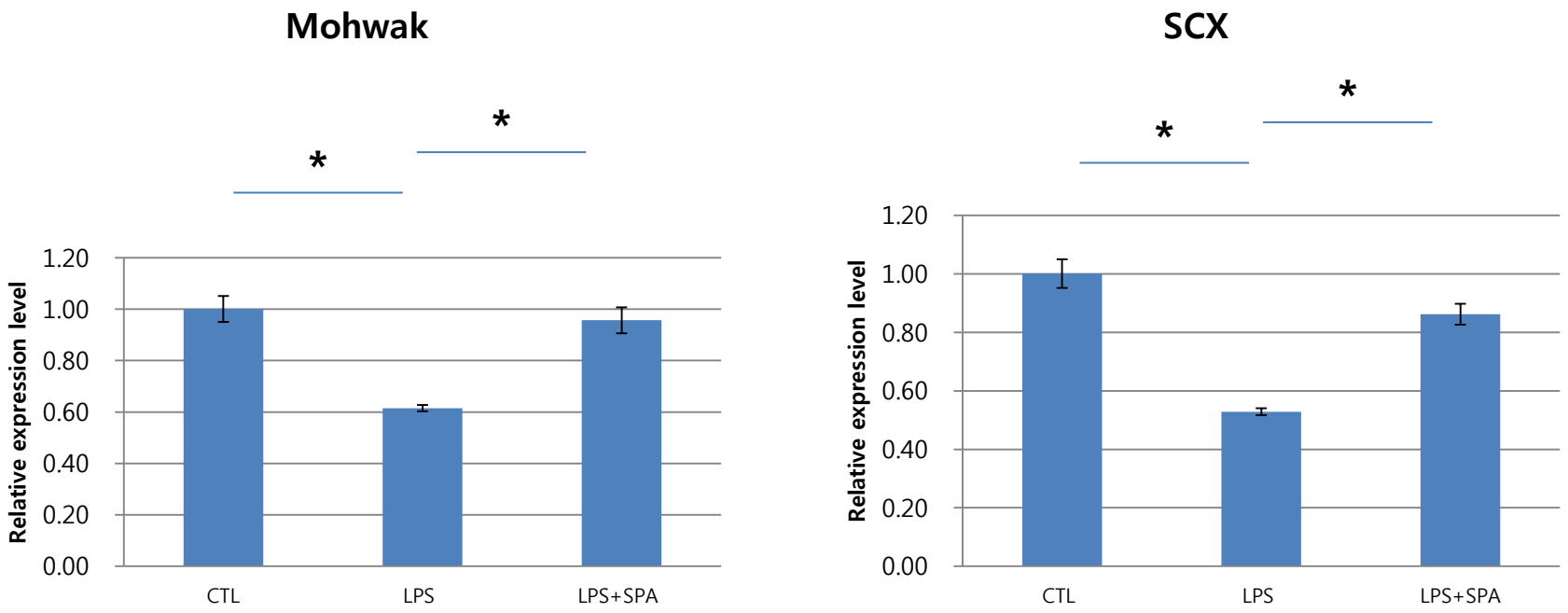
Ref: Inflammation and tendon healing -Parmis Blomgran-



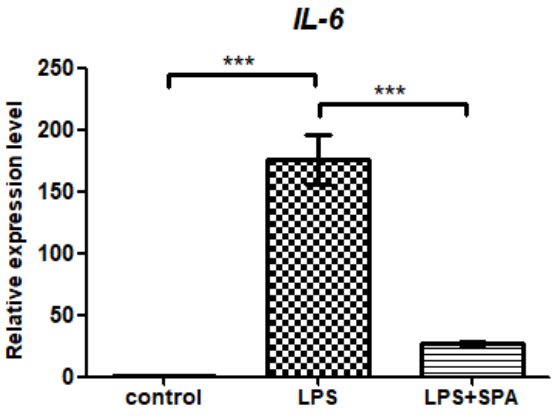
# Results

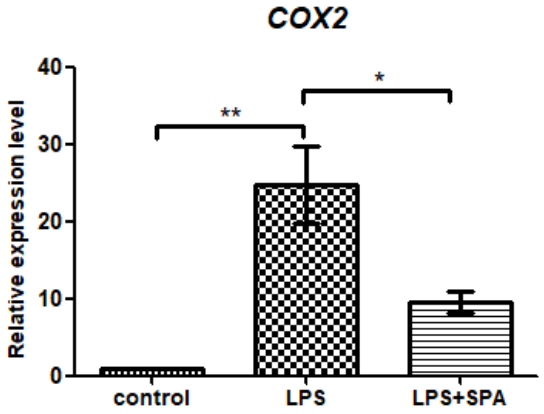
**Fig. 5. Effect of SP inhibitor on the tenocyte marker**

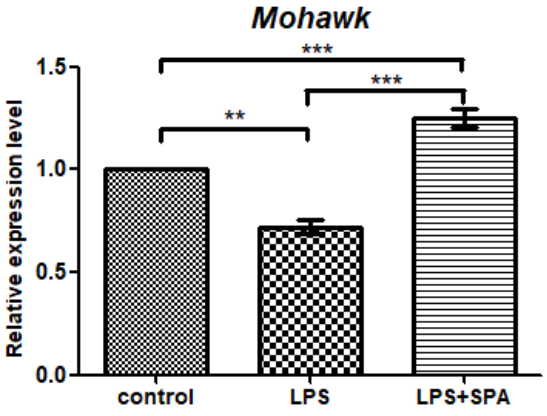
Tenocyte marke인 mohwak과 SCX의 expxression이 LPS를 처리하자 감소하엿음. 이것은 SP inhibitor 처리로 호전되었음. inflammatory tenocyte는 tenocyte 기능이 떨어짐. 이것이 marker expression 감소로 나타난다고 볼 수 있음. (이부분은 더 ref 찾아봐야 함.) 하지만 SP inhibito가 염증을 감소 시키며 tenocyte의 기능이 회복 되어 Mohawk, scx의 expression이 증가 함.

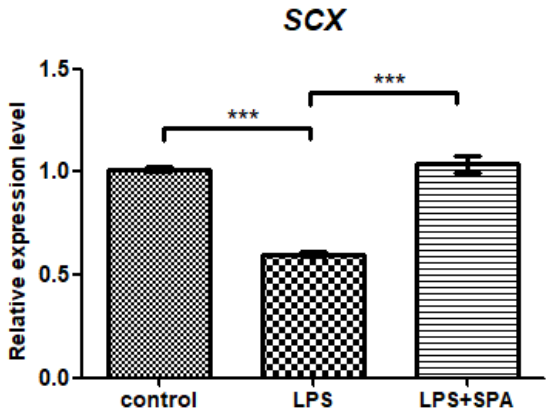


# 3. Anti-inflammatory effect of SP inhibitor on the inflamed tenocyte



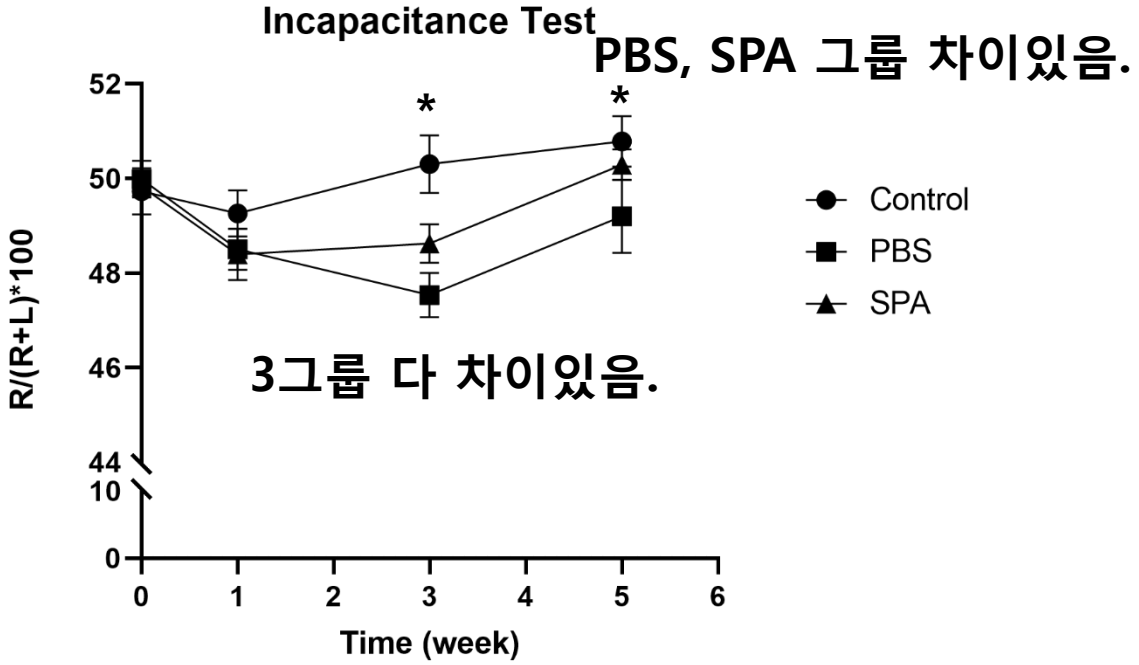



# Results

- **Fig. 6. Behavior test result**
- Incapacitance test was used to evaluate the changes of behavior after SP inhibitor injection. Both groups of healthy control and SP inhibitor injection displayed no significant difference in weight distribution between both hind-limbs. However, tendinopathy group had the lowest weight bearing in the affected limb. Dotted line indicates point of model induction. Hind limb weight difference was measured in grams (g). Data are displayed as mean ± SD. ***P < 0.001.



# Results

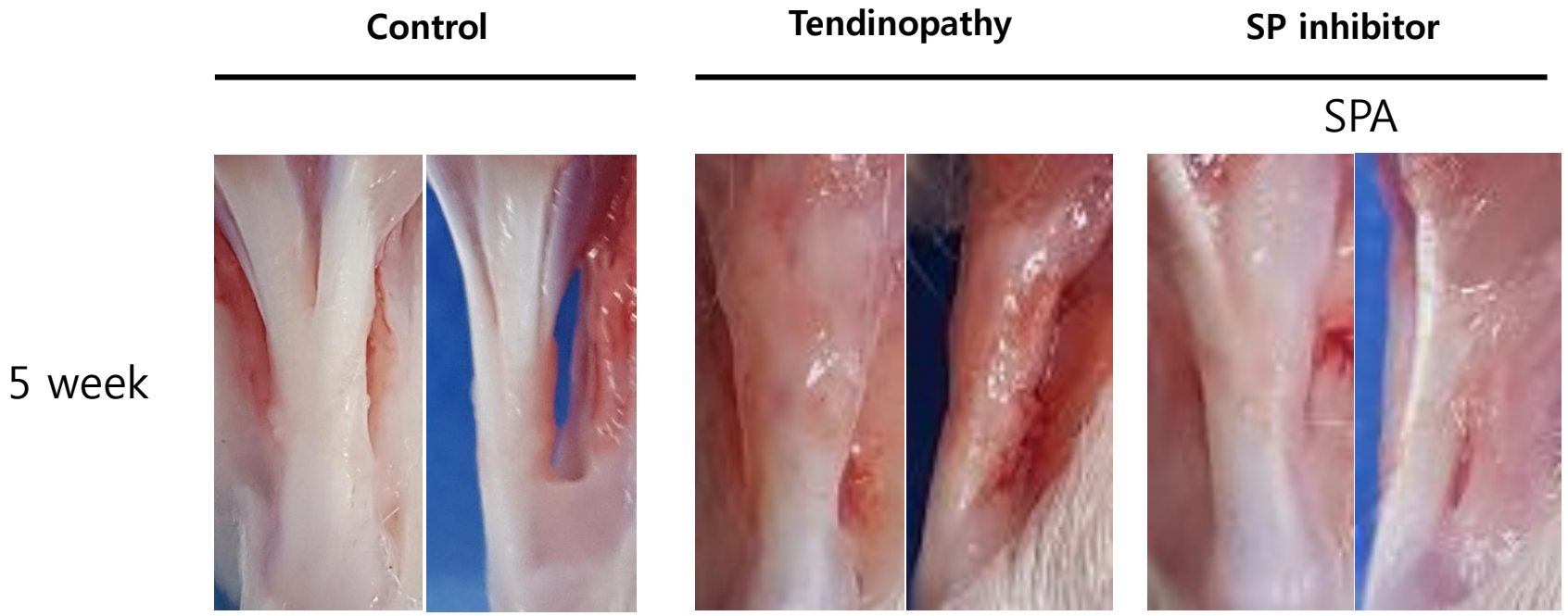
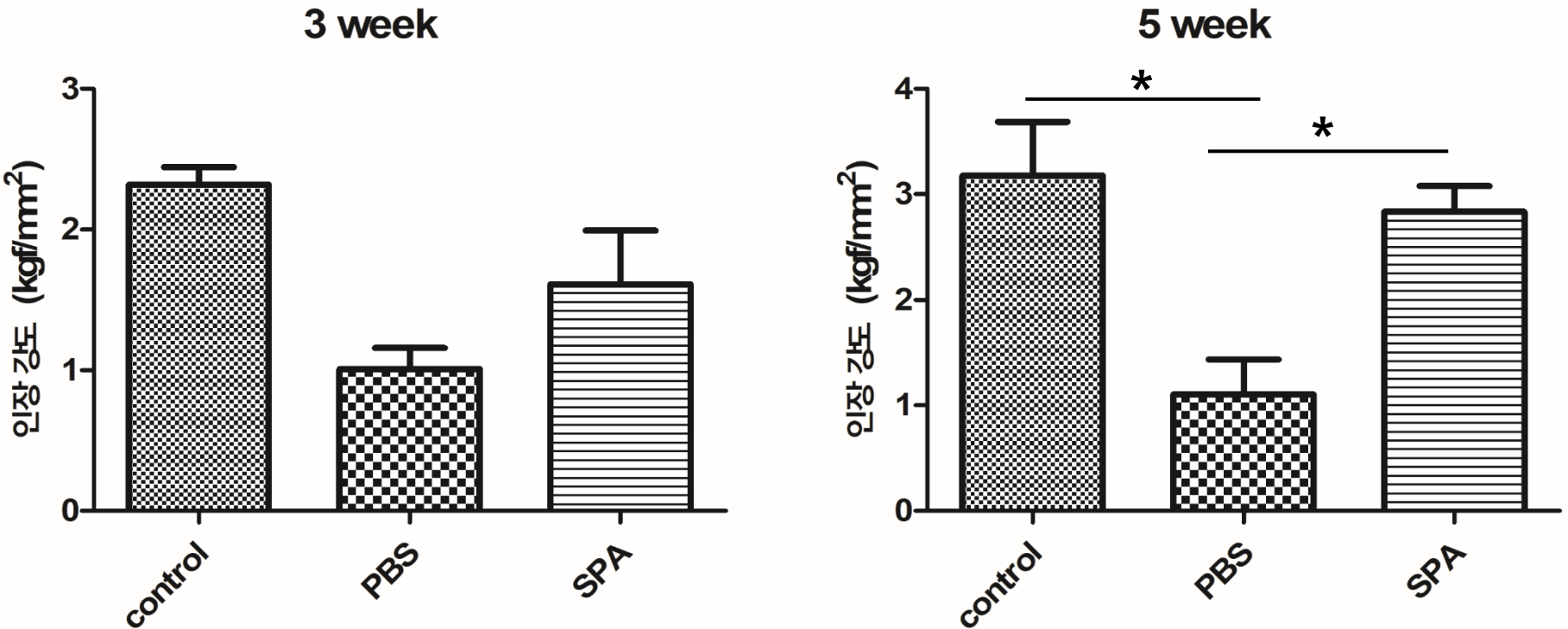



**Fig. 7. Gross photo of tendon (더 자세히)**
**Grossly collagenase group showed swelling and ..**
**But SP inhibitor group showed the smooth contour and shiny normal like tendon appearance**

# Results

## Fig. 8. Biomechanical test result

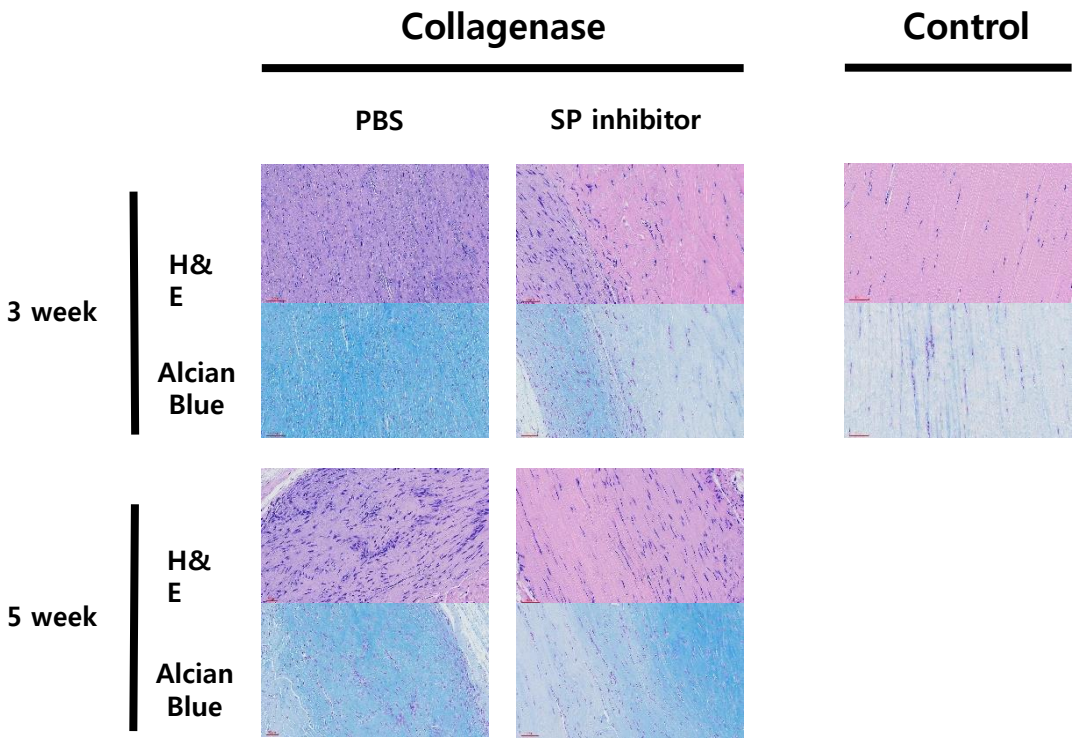
In order to further demonstrate the tendon restorative of SP inhibitor, we measured the tensile strengths of tendon tissues at 3 and 5 weeks. The tensile strengths of the tendon tissues in the collagenase-treated group were much lower than were those in the healthy control group. SP inhibitor increased the tensile strengths of the tendons compared to that of the collagenase-treated group at each time point. (** $p < 0.01$)



# Results (정: 사진 scanner 로 찍어서 김형경 선생님에게 보내서 해결)

**Fig. 9. Histopathological result**

- Histopathological examination with H & E staining and Alcian blue staining was performed to determine whether substance P inhibitor can improve tendon healing in tendinopathy tendon disruption.
- Normal tendons had well-aligned collagen fiber organization and no tendon disruption. In contrast, collagenase injection led to severe collagen matrix breakdown with an absence of well-aligned collagen fibers as shown in tendinopathy. However, in substance P inhibitor treatment group, there was only mild collagen matrix breakdown, suggesting that substance P inhibitor has the therapeutic effects on collagen disruption with much more aligned collagen fiber organization compared to that of collagenase group. There was no significant difference in the number of cell between collagenase injection and SP inhibitor.
- In Alcian blue staining, healthy control only had the scanty amount of ground substance, but alcian blue staining increased in the samples from collagenase injection, which meant the increased proteoglycans and glycosaminoglycans. The small focal proteoglycans and glycosaminoglycans deposition could be detected in the SP inhibitor. Scale bar = 50 μm


Collagenase
Control
PBS
SP inhibitor
3 week
H&
E
Alcian
Blue
5 week
H&
E
Alcian
Blue

# IHC

# Results (정: 사진 scanner 로 찍어서 김형경 선생님에게 보내서 해결)

**Fig. 10. Protein expression of NK1 receptor and inflammatory markers in each group.**

As expected, there was little staining for NK1 receptor, and IL-6 in healthy control compared to those of tendinopathy group. In contrast, samples of SP inhibitor injection showed decreased expression of all three proteins in comparison to the tendinopathy indicating SP inhibitor suppressed the inflammatory change in tendinopathy tendon. Scale bar = 100 μm

# Discussion

- Study summary 한문단
- SP가 tendinopathy 발생에 중요하다는 내용 간단 정리 한 문단
- Tenocyte에서 LPS를 처리하여 염증성 tenocyte를 만들어서 이후 SP inhibitor 처리 후 염증 감소에 대한 discussion 문장들, 이런 것들에 대한 기존의 논문들 정리, 즉 LPS로 염증을 일으키면 SP가 증가하나? SP inhibito가 COX2, il-6를 감소시키는 효과에 대한 연구가 있나?
- Ref: **Residual substance P levels after capsaicin treatment correlate with tendon repair.**
- **LPS → SP 증가 → IL-6 and COX2 증가**
- **이런 내용의 논문들 검색**

- Tenocyte에서 collagen 1, 3변화 에 대해
- Incapaciacne test 결과가 roburst 한차이가 없는것은, tendinopahty가 죽을 정도로 많이 아픈것이 아니기 때문 일듯.
- SP inhibitor는 이미 다른 용도로 사용되고 있다 하지만 tendinopathy에 사용되지 않았다는 내용. 향후 잘 design 된 임상 실험이 필요하겠다.

# Conclusion

- SP inhibitor는 tendon healing에 도움이 됨.

# SP+ PRP

# Study design

- Efficacy of autologous leukocyte-reduced platelet-rich plasma therapy for patellar tendinopathy in a rat treadmill model

# In vitro

- Healthy tenocyte (no treatment) → 증가 없음
- Inflammed tenocyte (IL-1b 처리, LPS) → SP, inflammatory mediator 증가
- Inflammed tenocyte (IL-1b 처리, LPS) + SP inhibitor → inflammatory mediator 증가 혹은 감소
- Inflammed tenocyte (IL-1b 처리, LPS) + SP inhibitor + PRP → inflammatory mediator 많이 감소

- LPS 2day + PRP 3day + SPA 2day
- LPS 농도 ($10^{-5}$M)

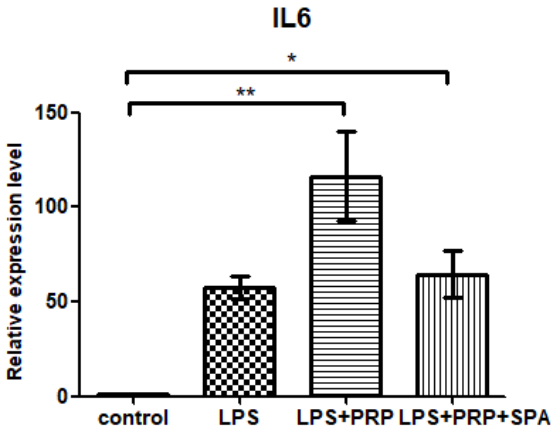
  PRP 농도 ($2x10^{7}$/ul, 10%)

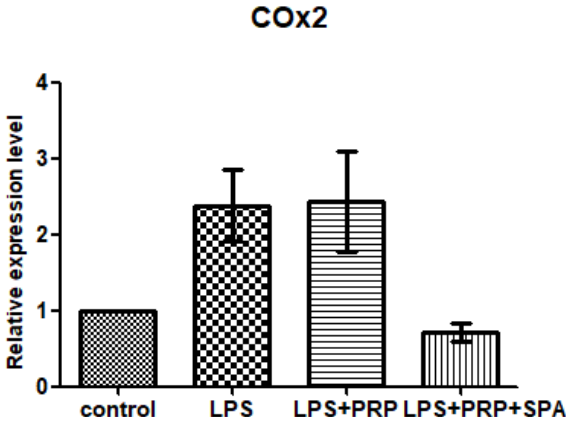
  SPA 농도 ($10^{-6}$M)

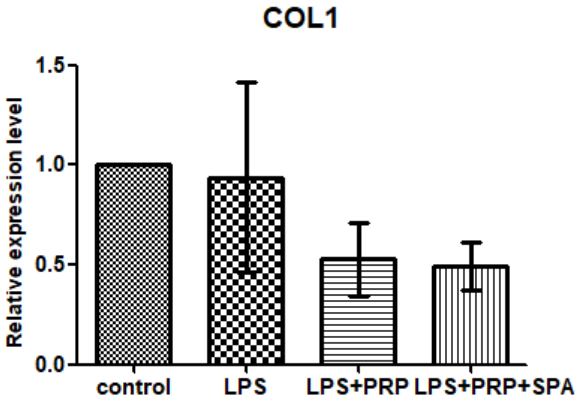
# PRP 조성

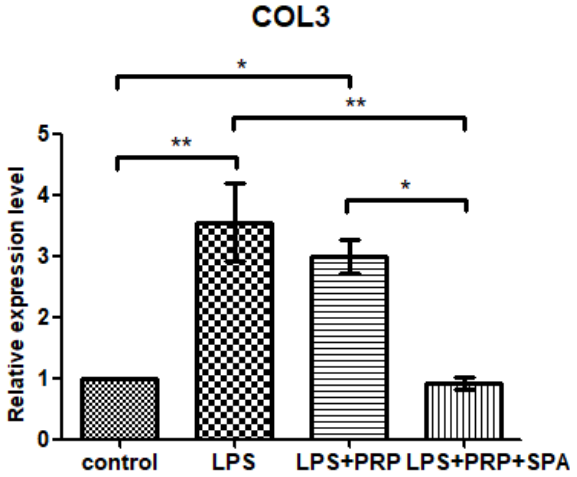
- 
- **Platelet rich plasma activates pro-inflammatory signaling pathways and induces oxidative stress in tendon fibroblasts**

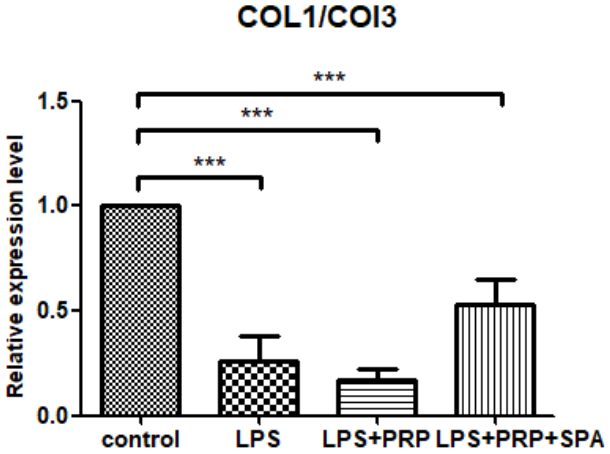
PCR

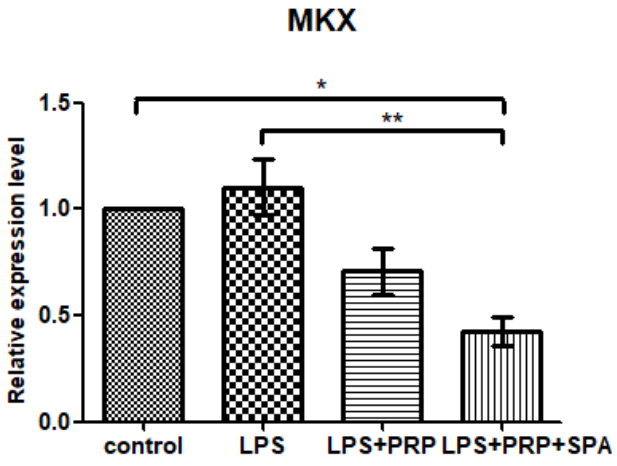

IL6
Relative expression level
150
100
50
0
*
**
control
LPS
LPS+PRP
LPS+PRP+SPA

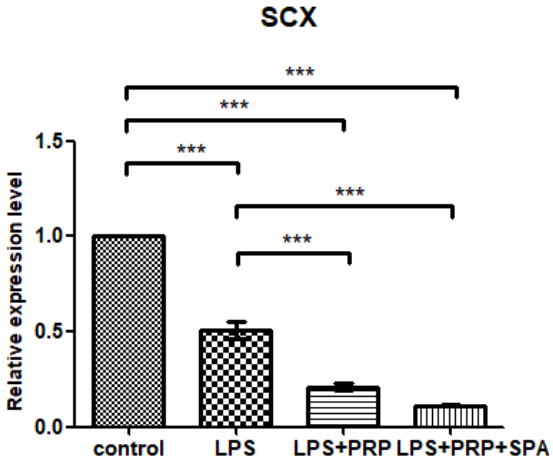

COx2
Relative expression level
4
3
2
1
0
control
LPS
LPS+PRP
LPS+PRP+SPA


COL1
Relative expression level
1.5
1.0
0.5
0.0
control
LPS
LPS+PRP
LPS+PRP+SPA


COL3
Relative expression level
5
4
3
2
1
0
*
**
**
*
control
LPS
LPS+PRP
LPS+PRP+SPA


COL1/COl3
Relative expression level
1.5
1.0
0.5
0.0
***
***
***
control
LPS
LPS+PRP
LPS+PRP+SPA


MKX
Relative expression level
1.5
1.0
0.5
0.0
*
**
control
LPS
LPS+PRP
LPS+PRP+SPA

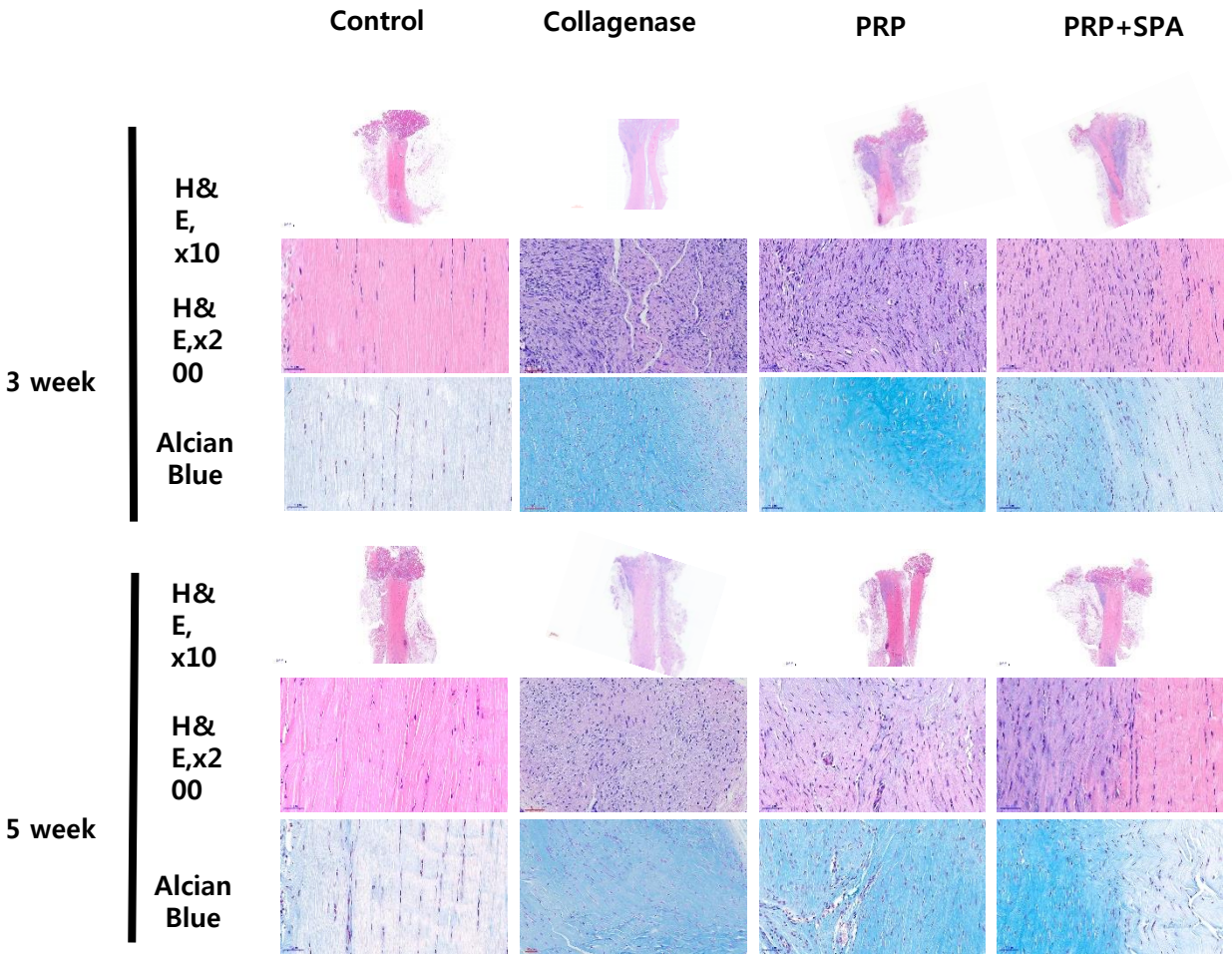

SCX
Relative expression level
1.5
1.0
0.5
0.0
***
***
***
***
***
control
LPS
LPS+PRP
LPS+PRP+SPA

# Incapaciance test

# Tension test

- Stem Cells and Platelet-Rich Plasma Enhance the Healing Process of Tendinitis in Mice

# Gross photo


Control
Collagenase
PRP
PRP+SPA
3 week
H&E, x10
H&E,x200
Alcian Blue
5 week
H&E, x10
H&E,x200
Alcian Blue

# IHC

# Histopathologic score

- Efficacy of autologous leukocyte-reduced platelet-rich plasma therapy for patellar tendinopathy in a rat treadmill model

- **Tendon Derived Stem Cells Promote Platelet-Rich Plasma Healing in Collagenase-Induced Rat Achilles Tendinopathy**