Possible Molecular Mechanism of Promotion of Repair of Acute Achilles Tendon Rupture by Low Intensity-pulsed Ultrasound Treatment in a Rat Model

T Kosaka, T Masaoka, K Yamamoto

ABSTRACT

Objective: *This study investigated the effect of Low Intensity-pulsed Ultrasound (LIPUS) on the repair process of ruptured Achilles tendon using a rat model and also examined the regulation of a biological molecule that may contribute to this* in vivo *and* in vitro.

Methods: *To investigate the effect of LIPUS and its biological mechanism of promoting Achilles tendon repair after acute injury, ninety-eight male Sprague-Dawley (SD) rats (mean body weight, 258 ± 9.8 g) aged 12 weeks were used in this study. To create the model, the Achilles tendon attachment site and musculotendinous junction were ruptured under direct vision. The leg on one side was exposed to LIPUS (frequency at 1.5 MHz, the repetition cycle at 1.0 kHz, the burst width at 200 msec and the power output at 45 mW/cm2), for 20 minutes daily with a 0.7 mm diameter probe.*

Results: *Low Intensity-pulsed Ultrasound treatment accelerated the repair of the Achilles tendon compared to the untreated group, judged by electron microscopy. Both cyclo-oxygenase (COX)-2* and EP4* expressions were over-expressed in the LIPUS treated group in the inflammatory period, and TGFβ1* expression was markedly induced in LIPUS treated groups followed by collagen I* and III* expression in the repair and reconstitution process.*

Conclusion: *These findings suggest that LIPUS is potentially able to accelerate the repair of acute ruptured Achilles tendon in several ways: by exaggerating inflammation by inducing COX-2 and EP4 and reconstituting tissue by inducing TGFβ1 followed by collagen I and III. (*:* $p < 0.05$, ***: 0.001)*

Keyword: Achilles tendon rupture, Collagen I, Collagen III, COX2, EP4R, LIPUS

Posible Mecanismo Molecular de Promoción de la Ruptura Aguda del Tendón de Aquiles por el Tratamiento de Ultrasonidos Pulsados de Baja Intensidad en un Modelo de Rata

T Kosaka, T Masaoka, K Yamamoto

RESUMEN

Objetivo: *Este estudio estuvo encaminado a investigar el efecto de los ultrasonidos pulsados de baja intensidad (LIPUS) sobre el proceso de reparación del tendón de Aquiles tras una ruptura, usando un modelo de rata. Asimismo, se examinó la regulación de una molécula biológica que puede contribuir a este proceso* in vivo *e* in vitro.

Métodos: *Con el fin de investigar el efecto de LIPUS y el mecanismo biológico por el cual este efecto promueve la reparación del tendón de Aquiles tras una lesión aguda, noventa y ocho ratas machos Sprague-Dawley (SD) (peso corporal promedio, 258 ± 9.8 g) de 12 semanas de edad fueron usadas en este estudio. Para crear el modelo, el sitio de ligazón microbiológica del tendón de Aquiles y la unión músculo-tendinosa fueron desgarrados bajo visión directa. La pierna de un lado fue expuesta a LIPUS (frecuencia de 1.5 MHz, ciclo de repetición de 1.0 kHz, ancho de ruptura de 200 msec, y potencia de salida de 45 mW/cm2), por 20 minutos diariamente con una sonda de 0.7 mm diámetro.*

Resultados: *El tratamiento de ultrasonidos pulsados de baja intensidad aceleró la reparación del tendón de Aquiles, en comparación con el grupo no tratado, según se apreció mediante el microscopio electrónico. Tanto la ciclo-oxygenasa (COX)-2* como las expresiones EP4* estuvieron sobe-*

From: Department of Orthopaedic Surgery, Tokyo Medical University, Tokyo, Japan.

Correspondence: Dr T Kosaka, Department of Orthopaedic Surgery, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku, Tokyo, Japan 160-0023. E-mail: tikosaka@h6.dion.ne.jp

expresadas en el grupo tratado con LIPUS en el periodo inflamatorio, y la expresión TGFβ1 fue marcadamente inducida en los grupos tratados con LIPUS seguidos por la expresión de colágeno I* y III* en el proceso de reparación y reconstitución.*

Conclusión: *Estos resultados sugieren que LIPUS puede potencialmente acelerar la reparación del tendón de Aquiles luego de un desgarramiento, de varias maneras: exagerando la inflamación mediante inducción de COX-2 y EP4 y reconstituyendo el tejido induciendo TGFβ1 seguido por colágeno I y III. (*:* $p < 0.05$, ***: 0.001)*

Palabras claves: Ruptura del tendón de Aquiles, colágeno I, colágeno III, COX2, EP4R, LIPUS

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INTRODUCTION

Pulsed ultrasound treatment has been well recognized to promote healing of bone fractures and has been applied in the clinical setting (1−4). There have been a number of reports suggesting that therapeutic ultrasound also promote tendon repairs. Enwemeka *et al* (5, 6) reported the beneficial effect of low intensity-pulsed ultrasound (LIPUS) on Achilles tendon repair in relation to its biomechanical properties on tenotomised tendon in their rabbit model. Yeung *et al* (7) also showed that LIPUS treatment of hemitenotomised Achilles tendon accelerated the repair, increased biomechanical strength, and generated better-aligned collagen fibres compared to untreated groups in their rat model. Thus, ultrasound treatment seems to be thera-peutically beneficial to the rupture site tendon functionally and structurally as seen in bone repair.

How does LIPUS promote tendon repair? In the case of bone repair, it has been reported that LIPUS stimulates the production of PGE2 by inducing cyclo-oxygenase (COX)-2 (8, 9) and also induces several growth factors including PDGF (8, 10), VEGF (11), TGFβ1 (12) and BMP-7 (12), which are all implicated in bone formation. On the other hand, although therapeutic ultrasound seems to promote the repair of Achilles tendon, the molecular mechanisms of this have not been well explored except one that shows that LIPUS stimulates the production of TGFβ1 followed by collagen I and III expression in isolated tenocytes *in vitro* (13, 14).

This study, therefore, investigated the effect of LIPUS on the repair process of Achilles tendon using a rat model and also examined the regulation of the biological molecule that may contribute to this *in vivo* and *in vitro*.

SUBJECTS AND METHODS

Ninety-eight male Sprague-Dawley (SD) rats (mean body weight, 258 ± 9.8 g) aged 12 weeks were used in this study. Ethical approval was obtained from the Tokyo Medical University ethical committee. All surgical procedures were carried out under general anaesthesia in a sterile condition with intraperitoneal injection of Nembutal (30 mg/kg). The skin posterior to both leg joints was shaved before surgery. To create the study model, the achilles tendon attachment site and musculotendinous junction were ruptured under direct vision. Only the skin in the equinus position was sutured and immobilized with a splint below the thigh.

Low intensity-pulsed ultrasound treatment was applied by using an ultrasound device (Rion Co. Ltd.). Immediately following surgery, the leg on one side was exposed to LIPUS (frequency at 1.5 MHz, the repetition cycle at 1.0 kHz, the burst width at 200 msec and the power output at 45 mW/cm2) for 20 minutes daily with a 0.7 mm diameter probe. After applying enough gel around the rupture site so that LIPUS transferred to the site efficiently, the device was immobilized with a tape. The LIPUS-treated or only probed group was depicted as US group and control groups, respectively. The rats were sacrificed after 0, 2, 5, 7, 14 and 28 days.

Tenocytes of Achilles tendon were isolated from the control side of rats that had been irradiated and were verified by staining with tenomodulin, which is one of the markers of tenocytes. Isolated tenocytes were cultured in a 10% FBS/DMEM in the presence of antibiotics. For the LIPUS treatment, cells at up to the 10^{th} passage were plated in 12-well culture plates and cultured until confluence. The LIPUS treatment was performed as described previously (14) with the same condition as described above. For rupture site tissue, rupture site Achilles tendons were dissected from the rats, snap-frozen in liquid nitrogen, ground into powder and homogenized under RNase-free conditions. The total RNA was extracted with TRIzol (invitrogen Japan KK, Tokyo, Japan) according to the manufacturer's instructions (8 rats per day). For tenocytes, cells were directly lysed in Isogen reagents (Nippon Gene Co. Tokyo, Japan) and total RNA was extracted according to the manufacturer's instructions.

Isolated total RNA (1 mg) was reverse transcribed using QuantiTech Reverse Transcription Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. A portion (an equivalent to 25 ng of total RNA) of the product of RT reactant was subjected to quantitative PCR for COX-2, EP1-4R, types I and III collagen and 18S rRNA using (Cat No. QT00374857 for rat EP1, Cat No. QT00183526 for rat EP2 and Cat No. QT00378378 for rat EP3, QT00377356 for rat EP4, Cat No. QT00187796 for rat TGFβ1, Cat No. QT01081003 for rat Type III collagen, < QUAGEN KK, Tokyo, Japan) and (COX-2: Forward/gtgaacatcaacc tgctgactgaac, Reverse/gcacggcagcag tcacatactta), (COX-1: Forward/gccgaggatgtcatcaaga,

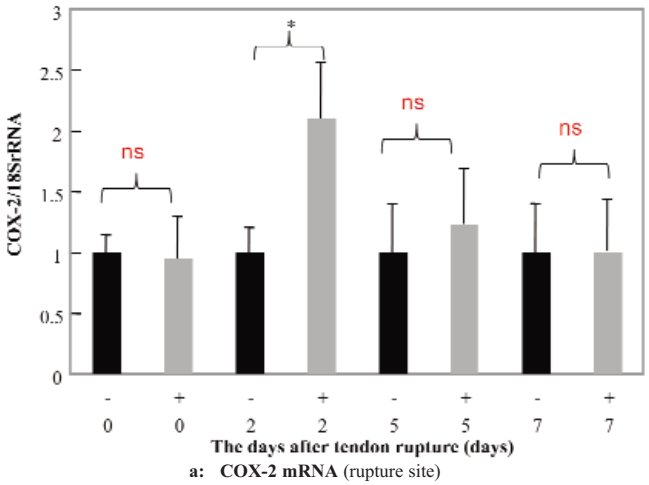
Reverse/gaactctaaagcatcgatgt cacca,) (Type 1 collagen: Forward/cggcagaagtctcaagatgg tggccg, Reverse/ctctcc gctcttccagtcaga,) (18SrRNA: Forward/aagtttcagcacatcct gcgagta, Reverse/ttggtgaggtcaatg tctgctttc,). The amplification cycle was performed at 94°C for 15 seconds (s), 55°C for 30s, and 72°C for 30s using ABI PRISM 7000 Sequence detection system (Applied Biosys-tems Japan Ltd, Tokyo, Japan). Obtained threshold cycle (CT) values of each sample were normalized by that of 18 seconds rRNA and relative expression level was expressed as mean value of control group at each time point as 1.

On day 28, the specimens were collected from the rupture site and immersed in a 0.1 M phosphate buffered solution with a 3% glutaraldehyde solution, and while being observed with a stereomicroscope, the longitudinal surface and transverse surface were finely cut and fixed overnight. Subsequently, it was placed in a 0.1 M phosphate buffered solution with ruthenium tetroxide (pH 7.4) for 2 hours (h), fixed at 4°C, washed with 10% simple sugar solution 3 times, and then block dyed in a 3% uranyl acetate aqueous solution for 1 h at room temperature. After being dehydrated and substituted with propylene oxide, the specimens were embedded in epoxide resin, and then ultrathin sections (0.1 nm) were cut with ultramicrotomy and observed using a transmission electron microscopy.

Data were analysed by one-way ANOVA followed by unpaired students *t*-test or Fishers PLDS test. Data are represented as mean ± SEM. Significance from the normal control was expressed as (*: $p < 0.05$, **: 0.001).

RESULTS

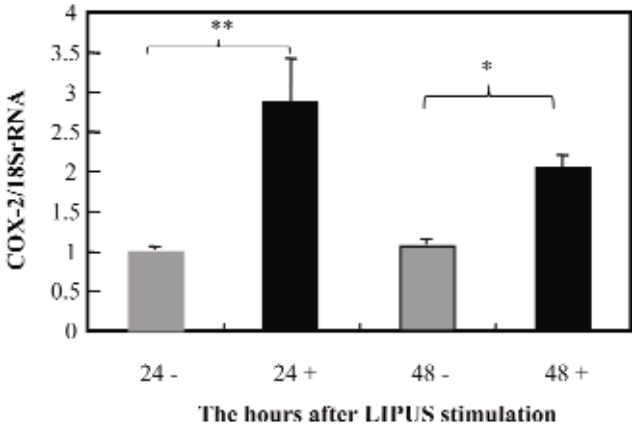
COX-2 expression was examined by quantitative-PCR. The expression of COX-2 at the rupture site was much higher in the US group compared to the control group (Fig. 1a). The



a: COX-2 mRNA (rupture site)

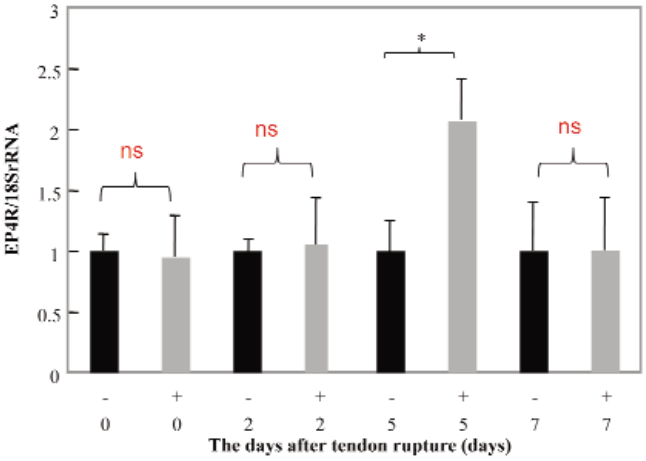
Fig. 1: Effect of LIPUS on COX-2 expression
a: (ruptured site tissue): Maximum and minimum COX-2 peaks were seen on day 2 and day 5, with significant expression on day 2 ($p < 0.05$).
(n = 8/day for control, n = 8/day for US)

significant difference was seen on day 2*. In order to clarify whether LIPUS directly affected tenocytes, isolated tenocytes were subjected to LIPUS treatment. LIPUS treatment stimulated tenocytes to induce COX-2 expression in a monolayer culture*, suggesting that tenocytes themselves are responsive to LIPUS treatment and this may in part be responsible for the upregulation of COX-2 *in vivo* (Fig. 1b).



b: **COX-2 mRNA (tenocytes culture)**

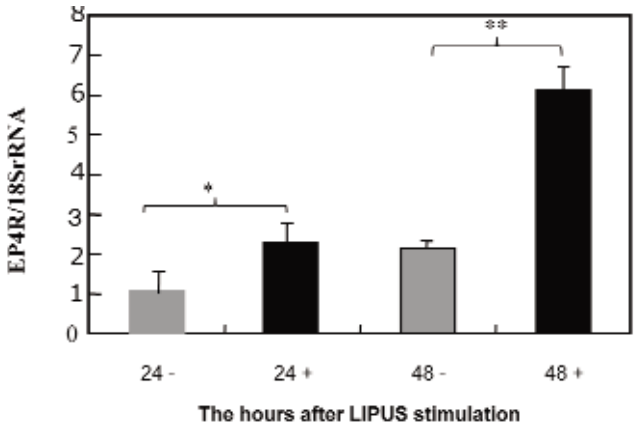
The expression of the receptors for PGE_2 (EP1-4) was also examined. Among 4 receptors, only EP4 was significantly regulated by LIPUS (data not shown). The difference of the expression level of EP4 was seen on day 2* and it became statistically significant on day 5 (Fig. 2a). This was also ob-



a: EP4R mRNA (rupture site)

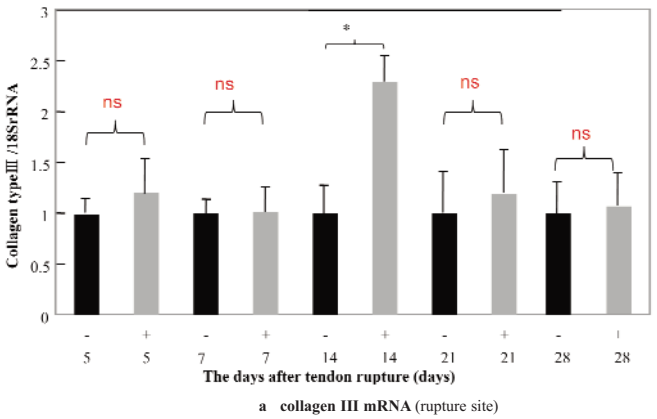
Fig 2: Effect of LIPUS on EP4 receptor expression
a: (ruptured site tissue) EP4R was significantly expressed on day 5 ($p < 0.05$).
(n = 8/day for control, n = 8/day for US)

served in isolated tenocytes culture (Fig. 2b). Taken together, these results suggest that LIPUS stimulates the expression of COX-2* and EP-4* at least in tenocytes and causes inflammation and swelling *in vivo*.



b: EP4R mRNA (tenocytes culture)

The tendon, including Achilles tendon, is mainly composed of collagen I and III fibres. The remodelling of collagen fibre is indispensable to the recovery of tendon function. Low intensity-pulsed ultrasound treatment increased both types I* and III* collagen expression in the area of transection 14 days after surgery when collagen remodelling was supposed to take place (Figs. 3a, 3b). As shown in Fig. 5, the



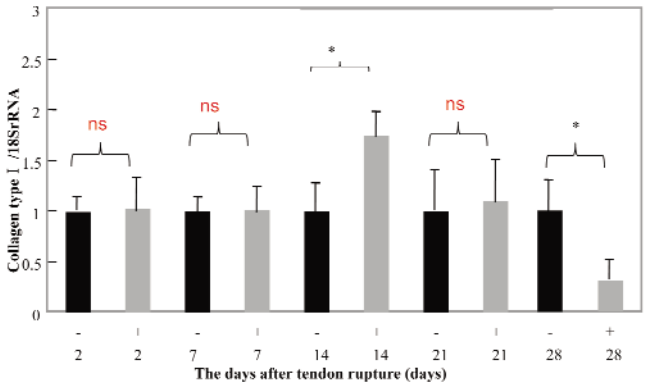
a collagen III mRNA (rupture site)

Fig 3: Effect of LIPUS on collagen expression
LIPUS treatment increased both types I and III collagen expression in the area of transection 14 days after surgery when collagen remodelling was supposed to take place. (n = 8/day for control, n = 8/day for US)

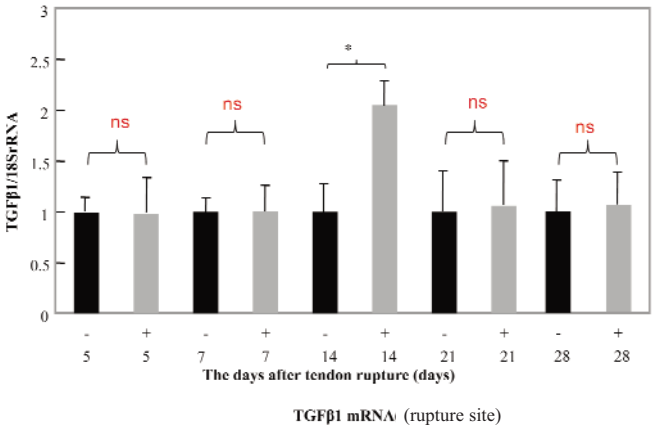
amount of collagen fibrils were rich in the US group, particularly, a marked increase was seen in the thicker fibrils in type I collagen. It is well known that TGFβ1 regulates collagen gene in skeletal tissues including bone and tendon, therefore TGFβ1 expression was also examined. Interestingly, TGFβ1 expression was higher in the US group at a similar time when collagen genes were upregulated* (Fig. 4).

DISCUSSION

The molecular mechanisms by which LIPUS accelerates Achilles tendon repair are not understood so far. The effect of LIPUS on the repair of bone fractures has been relatively



b collagen I mRNA (rupture site)

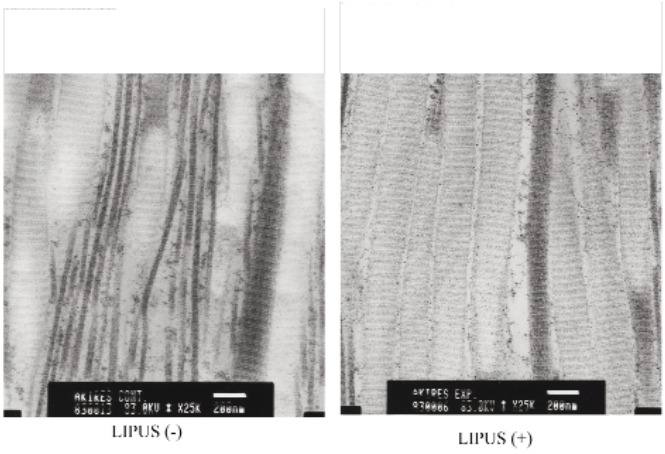


TGFβ1 mRNA (rupture site)

Fig. 4: Effect of LIPUS on TGFβ1 expression
LIPUS treatment increased TGFβ1 expression in the area of the rupture site 14 days after surgery when collagen remodelling was supposed to take place. (n = 8/day for control, n = 8/day for US)

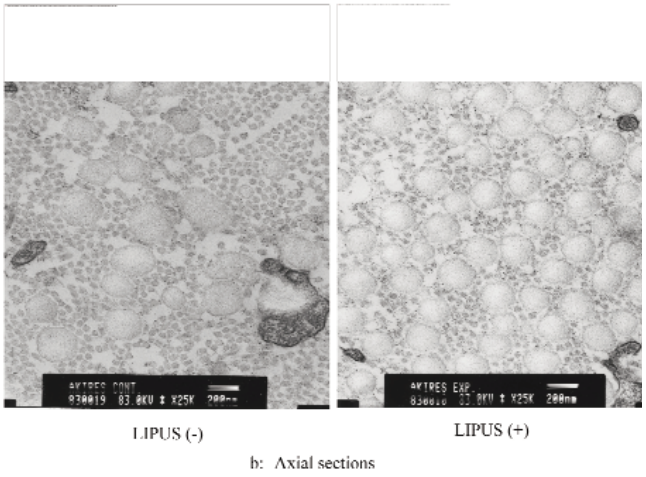
well studied. Low intensity-pulsed ultrasound treatment accelerates bone repair in experimental animal models and also in clinical settings. The molecular mechanism of this has been investigated by several groups using osteoblasts which are cells responsible for bone formation. Kokubu *et al* first reported that LIPUS stimulates mouse osteoblastic cell line, MC3T3-E1, to induce COX-2, followed by increase in PGE2 level (8). Thereafter, the numbers of biological mediators have been reported to be regulated by LIPUS in osteoblastic cells.

In the inflammatory periods, the COX-2 expression was significantly higher in the US group after 2 days of surgery where the transverse area was significantly increased over the control group. Although the difference of the transverse area increased up to 14 days, COX-2 expression came to a similar level to the control group by days 5 to 7. It was of interest that the expression of one of the receptors for PGE2, EP4, showed a greater expression compared to the control group around this period without affecting the other receptors (EP1-3, data not shown). Ligation of PGE2 to EP4, which is coupled to Gas, activates adenylyl cyclase and



LIPUS (-) LIPUS (+)

a: Sagittal sections



LIPUS (-) LIPUS (+)

b: Axial sections

Fig. 5: Electronic Microscope Imagery
a: sagittal, b: axial, by day 28, the amount of collagen fibrils had increased in the US group. Particularly, a marked increase was observed in the thicker fibrils seen.

results in increase in intracellular cAMP level (15). EP4 knock-out mice displayed reduced inflammation, as well as incidence and severity of collagen-induced arthritis (16). In addition, EP4 mediates skin inflammation after exposure to ultraviolet B light (17). Importantly, the repair of bone fracture was significantly delayed in EP4 knock-out mice compared to those of wild-type mice (18) and a selective EP4 antagonist significantly delayed the ulcer healing induced by thermo-cauterization in both mouse and rat (19). Therefore, EP4 seems to be an important receptor not only for mediating inflammation but also for repairing tissues. Higher levels of EP4 expression seen in the US group may explain the prolonged inflammatory period and enlarged transverse area. In the repair and reconstitution period, the gap was filled with collagen fibres in both groups whereas they were thicker, richer and well aligned in the US group as determined by electron microscopy. Further, electron microscopy confirmed that type I collagen in the US group was indeed much thicker and denser than that of the control group. These observations were consistent with the previous study reported by Yeung *et al* (7). The cellular mechanism underlying the anabolic effect of LIPUS has been postulated. It has been reported that ultrasound increases the proliferation and also stimulates the expression and production of TGFβ1 and collagen I and III in isolated tendon cells (13, 14). One can hypothesize that the beneficial anabolic effect seen in the repair and reconstitution periods associates with the expres-sion of TGFβ1 *in vivo*. Our *in vivo* data confirmed that the expression of TGFβ1 was significantly higher on day 14 in the US group and both collagen I and III expression followed the growth factor. Although the cells responsible for the expression of TGFβ1 and collagens are not clear, tenocytes, at least, do sense the ultrasound and induce anabolic phenotype in these periods.

It has been reported that selective COX-2 inhibitor, which is routinely used for patients to inhibit inflammation, attenuates tenocyte proliferation and migration without affecting collagen I and III expression *in vitro* (20).

In conclusion, the present study demonstrated the therapeutically beneficial effects of LIPUS on repairing acute Achilles tendon rupture in rat model and also demonstrated, for the first time, that molecular mechanisms are multifarious; they stimulate COX-2 and EP4 expression in the inflammatory period and induce TGFβ1 followed by collagen I and III.

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