Protective Effects of Extracorporeal Shockwave Therapy on the Degenerated Meniscus in a Rat Model

Abstract

Background

Loss of the meniscus function in association with degenerative changes affects the development and progression of knee osteoarthritis, for which there is currently no effective treatment. Extracorporeal shockwave therapy (ESWT) is an established treatment for musculoskeletal disorders. However, the therapeutic effect of ESWT on meniscal degeneration remains unclear.

Purpose

To evaluate the therapeutic effect of ESWT on the degenerated meniscus of the anterior cruciate ligament transection (ACLT) model.

Study Design

Controlled laboratory study.

Methods

Twelve-week-old male Wistar rats were randomly assigned to three groups (normal, ESWT–, and ESWT+). Unilateral ACLT of the right knee was performed in the latter

two groups. At 4 weeks after ACLT, the ESWT+ group received 800 shockwave impulses at an energy flux density of 0.22 mJ/mm² in a single session. Histological changes were examined in the posterior portion of the medial meniscus after ESWT (n=15 per group). In addition, we performed real-time polymerase chain reaction (PCR) after ESWT (n=5 per group) to analyze the expression of Connective tissue growth factor/CCN family member 2 (CTGF/CCN2), SRY (sex determining region Y)-box 9, vascular endothelial growth factor α, aggrecan, collagen type 1 alpha 2 (Col1a2), and collagen type 2 alpha 1 (Col2a1). Immunohistochemistry was used to analyze the expression of CTGF/CCN2 and Ki-67 (n=5 per group) after ESWT.

Results

The meniscal histopathological score at 4 weeks after ACLT was significantly higher than that in the normal group, and the score in the ESWT+ group was significantly lower than that in the ESWT– group at 4 and 12 weeks after ESWT. In addition, real-time PCR revealed that the mRNA expression of CTGF/CCN2 and Col2a1 decreased 4 weeks after ACLT. In the ESWT+ group, real-time PCR revealed that the mRNA expression of CTGF/CCN2 increased 24 h after ESWT, and the expression of Col2a1 increased 4 weeks after ESWT (all significant data were $P<0.05$). The ratio of CTGF/CCN2-positive cells and Ki67-positive cells was significantly higher in the

ESWT+ group after ESWT.

Conclusion

The present study revealed that ESWT might suppress ACLT-induced meniscus degeneration by stimulating cartilage repair factors and inducing collagen type 2.

Clinical relevance

ESWT can be an effective treatment to protect the degenerated meniscus in a rat model of ACLT.

Key terms

extracorporeal shockwave therapy (ESWT); meniscal degeneration; cartilage repair factor; CCN2; collagen type 2

What is known about the subject:

ESWT is known to protect the cartilage of the knee and prevent progression of osteoarthritis, in addition to promoting the healing of meniscus tears.

What this study adds to existing knowledge:

ESWT can therefore be an effective option for the treatment of degenerated menisci after an anterior cruciate ligament tear.

Introduction

Knee osteoarthritis is a clinically common disease that affects a large number of patients and has a significant impact on socioeconomics and patient well-being[12]. The degenerative changes and damage of the cartilage and meniscus are related to the development and progression of knee osteoarthritis[1,3-5,22]. The meniscus has essential roles in joint lubrication, shock absorption during dynamic loading, and stability of deficient cruciate ligaments[6,19,23]. Because medial meniscus contact force and internal hoop tension increase in anterior cruciate ligament-deficient knees, meniscal injury is thought to be associated with chronic anterior cruciate ligament tears[10,27]. In knee osteoarthritis induced by anterior cruciate ligament transection, meniscal degeneration precedes cartilage degeneration[3]. Therefore, the prevention and treatment of meniscal degeneration and damage are critical to prevent the development and progression of knee osteoarthritis after an anterior cruciate ligament tear. However, there is currently no effective treatment for meniscal degeneration.

Extracorporeal shock wave therapy (ESWT) has therapeutic effects on musculoskeletal disorders[25]. ESWT promotes biological processes, including tissue regeneration, wound healing, angiogenesis, bone remodeling, anti-inflammation, and chondroprotective effects[2,29,31-33]. Previous studies have revealed that ESWT

72 demonstrates chondroprotective effects by promoting the expression of collagen type 2
73 and osteogenesis growth factors, angiogenesis, subchondral bone remodeling, and anti-
74 inflammation[25,29,32,33]. On the other hand, in the repair process of meniscus tears, ESWT
75 affected the activation of factors related to meniscal healing and the activation of cell
76 proliferation, thereby promoting the repair process[11]. However, the therapeutic effect of
77 ESWT on the degenerated meniscus remains unknown.
78 This study aimed to investigate the reparative or protective effects of ESWT on
79 the degenerated meniscus after an anterior cruciate ligament tear. In the present study,
80 the frequently used anterior cruciate ligament transection (ACLT) model—in the
81 meniscus is not damaged during surgery—was used to achieve this objective. We
82 hypothesized that ESWT would suppress the progression of meniscal degeneration by
83 activating meniscal healing factors.
84

Methods

Animals

The animal experimentation protocol (20-038) in the present study was approved by the Animal Care and Experimentation Committee of Gunma University. All efforts were made to minimize the suffering and the number of animals used in this study. Eighty medial menisci of 12-week-old male Wistar rats (body weight, 240-280 g) that were purchased from Japan SLC (Hamamatsu, Japan) were used in this study. All rats were housed at the Biological Resource Center under controlled temperature (24°C) and illumination with a 12-hour light and dark cycle, with *ad libitum* access to food and tap water.

Surgical Procedure

The rats were divided into three groups (normal, ESWT–, and ESWT+). The normal group stayed without surgery. The two other groups underwent ACLT via an incision on the medial parapatellar approach. The rats in the surgical groups were anesthetized with an intraperitoneal injection of ketamine and xylazine (60 mg ketamine/kg body weight and 12 mg xylazine/kg body weight). According to the method of a previous study[3,15], the right knee joint was opened via a medial parapatellar approach with the patella

dislocated laterally. Then, the anterior cruciate ligament was identified and transected with a scalpel without damaging the cartilage or meniscus. The articular capsule and skin were closed with interrupted sutures using 5–0 silk thread. The rats in both groups were allowed unrestricted cage activity. None of the rats showed severe activity limitation after the surgery. None of the rats had infected wounds.

ESWT application

According to the method of a previous study, the ESWT+ group received 800 impulses of shockwave at 0.22 mJ/mm² energy flux density under general anesthesia in a single session at 4 weeks after surgery (Dornier MedTech; Dornier ARIES Vet)[11,31]. A single type of ESWT at a single time point induced the activation of meniscal repair and cartilage protection[11,31]. The ESWT probe was applied slightly behind the medial joint line in full extension under anesthesia with an intraperitoneal injection of ketamine and xylazine (60 mg of ketamine/kg body weight and 12 mg of xylazine/kg body weight). The focus of shockwave treatment was the medial meniscus posterior segment. Ultrasound gel was applied to the skin in contact with the shockwave probe.

Sample Collection and Preparation

The rats were euthanized at 24 h and 2, 4, and 12 weeks after ESWT. Fifty medial menisci were subjected to histological examinations, and 30 were analyzed by real-time polymerase chain reaction (PCR). The medial meniscus was harvested from the knee joint, and the surrounding synovial membrane was removed. The harvested menisci were immediately immersed in formalin for 24 h for histological evaluation. They were immersed in a neutral decalcification solution (Yuaikasei) for five days and embedded in paraffin. The meniscus specimens for real-time PCR were frozen in liquid nitrogen immediately after collection and stored at -80°C until use.

Histologic Evaluation of Meniscal Degeneration

We used specimens obtained 4 weeks after ACLT to evaluate the degenerative effect of ACLT on the medial meniscus. We used specimens obtained at 2, 4, and 12 weeks after ESWT for evaluation in the ESWT– group and the ESWT+ group (n=5 per group) and analyzed the medial meniscus posterior segment. The paraffin blocks were cut at 2.5 μm, and serial sections perpendicular to the posterior segment of the meniscus were stained with hematoxylin-eosin and Safranin O-Fast Green. We determined sections perpendicular to the posterior segment of the meniscus by measuring the length of the posterior segment and setting the evaluated section as the median value from the

beginning of the cut. We determined the meniscal degenerative score, which graded i) surface integrity, ii) cellularity, iii) matrix/fiber organization and collagen alignment, and iv) Safranin-O staining intensity. The score ranges from 0 to 18 points (this total score can be converted to a grade as follows: G1 = 0–4 (normal tissue), G2 = 5–9 (mild degeneration), G3 = 10–14 (moderate degeneration), and G4 = 15–18 (severe degeneration)[15,22]. Two orthopedic surgeons independently assessed ten specimens to calculate the interobserver intraclass correlation coefficient. The interobserver intraclass correlation coefficient intraobserver intraclass correlation coefficient were 0.84 and 0.90, respectively.

Immunohistochemical Analyses

We used specimens obtained 2 and 4 weeks after ESWT for evaluation in the normal, ESWT–, and ESWT+ groups (n=5 per group). After deparaffinization and rehydration, 2.5-mm-thick sections were prepared with a streptavidin-biotin-peroxidase system kit (Histofine; Nichirei) and chromogen (diaminobenzidine). Specimens were stained with rabbit polyclonal anti-Ki67 antibody (concentration 1:200; Novus) to analyze the proliferation rate of meniscal cells, anti-Connective tissue growth factor/CCN family member 2 (CTGF/CCN2) antibody (concentration 1:800; Abcam) to analyze the change

157 in the ratio of cells expressing cartilage-repairing factor, and anti-collagen type 2
158 antibody (concentration 1:200; Abcam) to analyze changes in the expression of collagen
159 type 2.

160 We identified immunoactivity from five frames of sections obtained from the
161 same specimen. We analyzed images using a cell counter plugin software program (Fiji
162 ImageJ). For quantitative measurements, percentages refer to the sum of the positively
163 stained cells over the sum of the total cells counted in all five frames[30,32]. In addition,
164 ten frames of images from randomly selected specimens were independently assessed
165 by two orthopedic surgeons to calculate the interobserver intraclass correlation
166 coefficient. The interobserver intraclass correlation coefficient and intraobserver
167 intraclass correlation coefficient were 0.84 and 0.86, respectively.

168

169 **Real-time PCR**

170 A 3×3 mm piece of the posterior segment of the medial meniscus was resected from the
171 ESWT– and ESWT+ groups at 24 h and 4 weeks after ESWT. In the normal group, a
172 3×3 mm piece of the posterior segment of the medial meniscus was harvested from rats
173 of the same age (n=5 per group). For this experiment, a bead crusher μT-12 (Taitec) was
174 used to homogenize the piece of the meniscus. An RNeasy Mini Kit (Qiagen) was used

175 to extract the total RNA. cDNA was synthesized from isolated total RNA with a
176 ReverTra Ace® qPCR RT Kit (Toyobo). Then, quantitative real-time PCR was
177 performed according to the instructions of SYBR Green Real-time PCR Master Mix
178 (Toyobo). A StepOne Real-Time PCR System (Applied Biosystems) was used to
179 perform quantitative real-time PCR. We measured the mRNA levels of CTGF/CCN2,
180 SRY (sex determining region Y)-box 9 (SOX9), vascular endothelial growth factor α
181 (VEGF-α), aggrecan (Acan), collagen type 1 alpha 2 (Col1a2), and collagen type 2
182 alpha 1 (Col2a1). Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were
183 used as an internal control. The quantified relative expression of these genes was
184 normalized to GAPDH by the delta-delta CT method. The nucleotide sequences of the
185 primers are shown in Table 1.

186 **Table 1.** Primers

Gene	Primer Sequence
GAPDH	
Forward	5’-GTCTTCACTACCATGGAGAAGG-3’
Reverse	5’-TCATGGATGACCTTGGCCAG-3’
CTGF/CCN2	
Forward	5’-CCACCCGAGTTACCAATGAC-3’
Reverse	5’-GTGCAGCCAGAAAGCTCA-3’
SOX9	
Forward	5’-AGACCAGTACCCGCATCT-3’
Reverse	5’-CGCTCCGCCTCCTCCAC-3’
VEGF-a	

Forward	5’-TTCAGAGCGGAGAAAGCATT-3’
Reverse	5’-GAGGAGGCTCCTTCCTGC-3’
ACAN	
Forward	5’-TTGGAGCCGGAGACGACAGA-3’
Reverse	5’-AGAGGCAGAGGGACTTTCGGT-3’
COL1A2	
Forward	5’-CCGTGCTTCTCAGAACATCA-3’
Reverse	5’-CTTGCCCCATTCATTTGTCT-3’
COL2A1	
Forward	5’-TTCCTCCGTCTACTGTCCACTGA-3’
Reverse	5’-CTACATCATTGGAGCCCTGGAT-3’

Statistical Analysis

All data are expressed as the mean ± standard deviation (SD). The Kruskal–Wallis test followed by the Dunn-Bonferroni post hoc test method was used to evaluate differences among the three groups. The Mann–Whitney U test was used to evaluate the differences between the two groups. All statistical analyses were performed using SPSS version 25.0 (IBM Japan, Tokyo, Japan). *P* values of <0.05 were considered to indicate statistical significance.

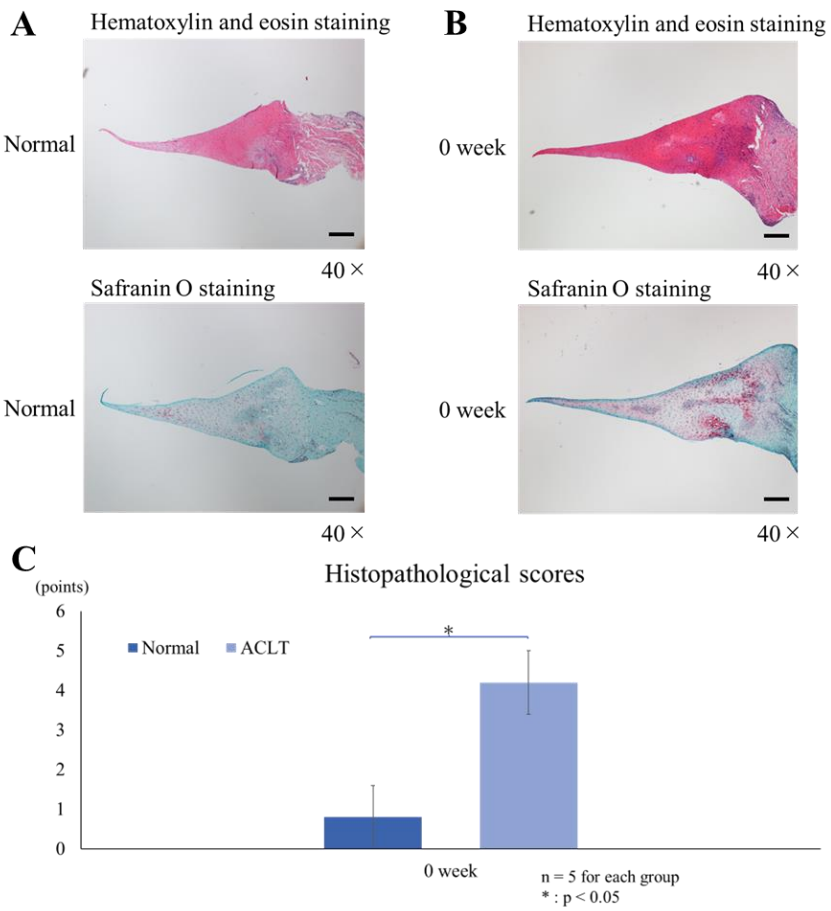
Results

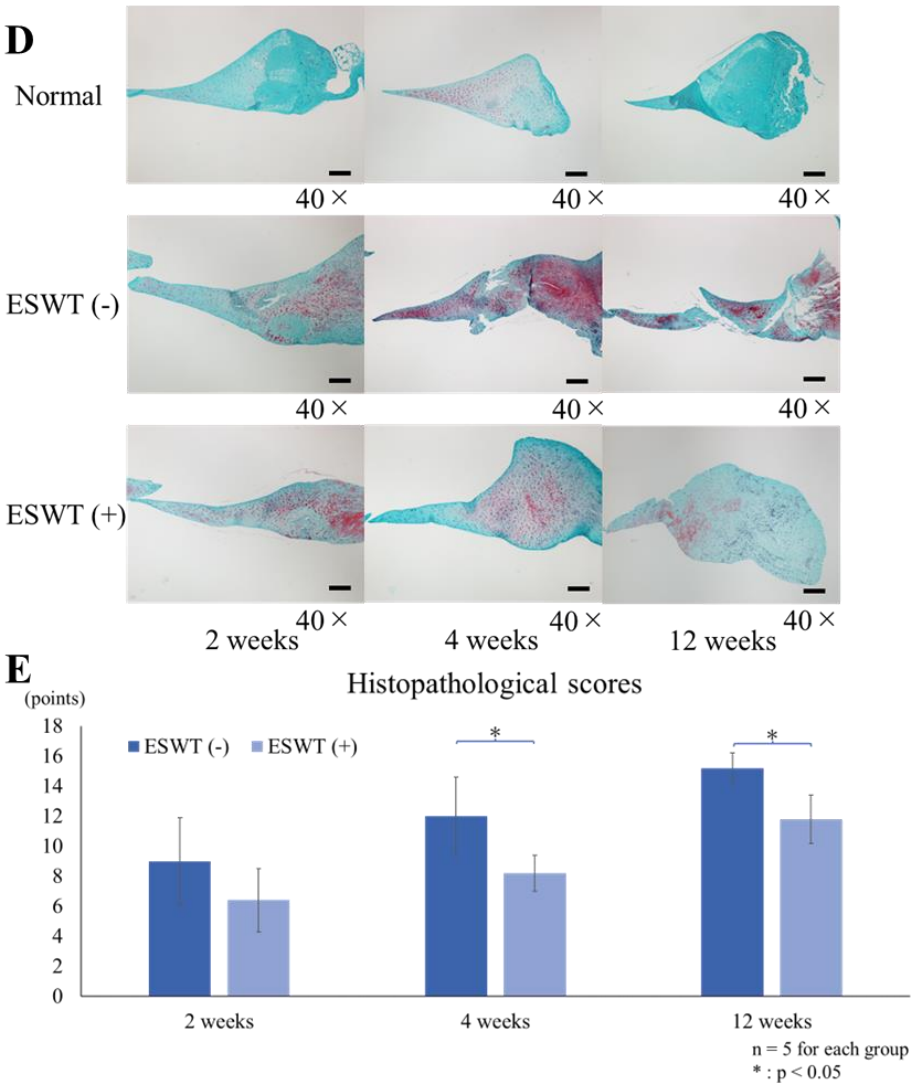
Histopathological Analyses

The histological examination of the normal meniscus with HE staining and safranin O staining showed that the meniscus surface was smooth, with well-defined cell distribution and density, and slight safranin O staining in the inner region (Figure 1A). In the normal group, no significant degenerative changes were observed over time with aging during the observation period. At 4 weeks after ACLT (i.e., 0 weeks post-ESWT), the meniscal surface was smooth. However, areas of hypercellularity were observed in the outer regions, and the safranin O staining intensity was slight (Figure 1B). From 4 weeks post-ACLT, the ESWT– group showed an increase in tears from the meniscal surface to inside the meniscus, an increase in areas of hypocellularity, more disorganized extracellular matrix, and stronger safranin O staining over time (Figure 1D). From 4 weeks post-ACLT, the ESWT+ group showed a gradual increase in the number of hypocellular areas and a slight disruption in extracellular matrix organization, but the changes, including the damage to the meniscus surface, occurred relatively gradually (Figure 1D).

The meniscal histopathological scores are shown in Figure 1C, E. The scores of the normal group were significantly lower than those of the ESWT– group at 0 weeks

(mean ± SD: 0.8 ± 0.8 vs. 4.2 ± 0.8, p=0.008) (Figure 1C). The scores in the normal group did not increase over time with aging during the observation period. However, the scores gradually increased in the ESWT+ and ESWT– groups. At 2 weeks, the scores of the ESWT+ and ESWT– groups did not differ to a statistically significant extent. However, the score of the ESWT+ group was significantly lower than that of the ESWT– group at 4 weeks (mean ± SD: 8.2 ± 1.2 vs. 12.0 ± 2.6, p=0.016) and 12 weeks (11.8 ± 1.6 vs. 15.2 ± 1.0, p=0.008) (Figure 1E).





222

223 **Figure 1.** Histopathological findings of meniscus degeneration and histopathological

224 scores. (A) Histopathological findings of the normal meniscus and (B) meniscus at 4

225 weeks after anterior cruciate ligament transection (ACLT) and 0 weeks after

226 extracorporeal shockwave therapy (ESWT) with hematoxylin and eosin (HE) staining

227 and safranin O staining. (C) The histopathological scores at 4 weeks after ACLT and 0

228 weeks after ESWT. (D) Safranin O staining in the normal, ESWT–, and ESWT+ groups

229 at 2, 4, and 12 weeks after ESWT. (E) The histopathological scores at 2, 4, and 12

230 weeks after ESWT. Data are expressed as the mean ± standard deviation. P values were
231 determined with the Mann–Whitney U test. *P<0.05 (n=5 per group). Bar=200μm
232
233 **Immunohistochemical Analysis**
234 The medial meniscus posterior segment in the normal, ESWT– and ESWT+ groups was
235 subjected to immunostaining with an anti-CTGF/CCN2 antibody at 2 and 4 weeks after
236 ESWT (Figure 2A). The ratio of CTGF/CCN2-positive cells to all cells is shown in
237 Figure 2B. This ratio did not change at any of the observation time points in the normal
238 group (47.8% and 45.8%, respectively). The overall positive cell ratio increased to
239 71.1% at 4 weeks in the ESWT+ group, while the positive cell ratio decreased to 25.6%
240 at 4 weeks in the ESWT– group. In the ESWT+ group, this ratio was significantly
241 higher than that in the ESWT– group at 2 weeks (p=0.003) and 4 weeks (p=0.002).

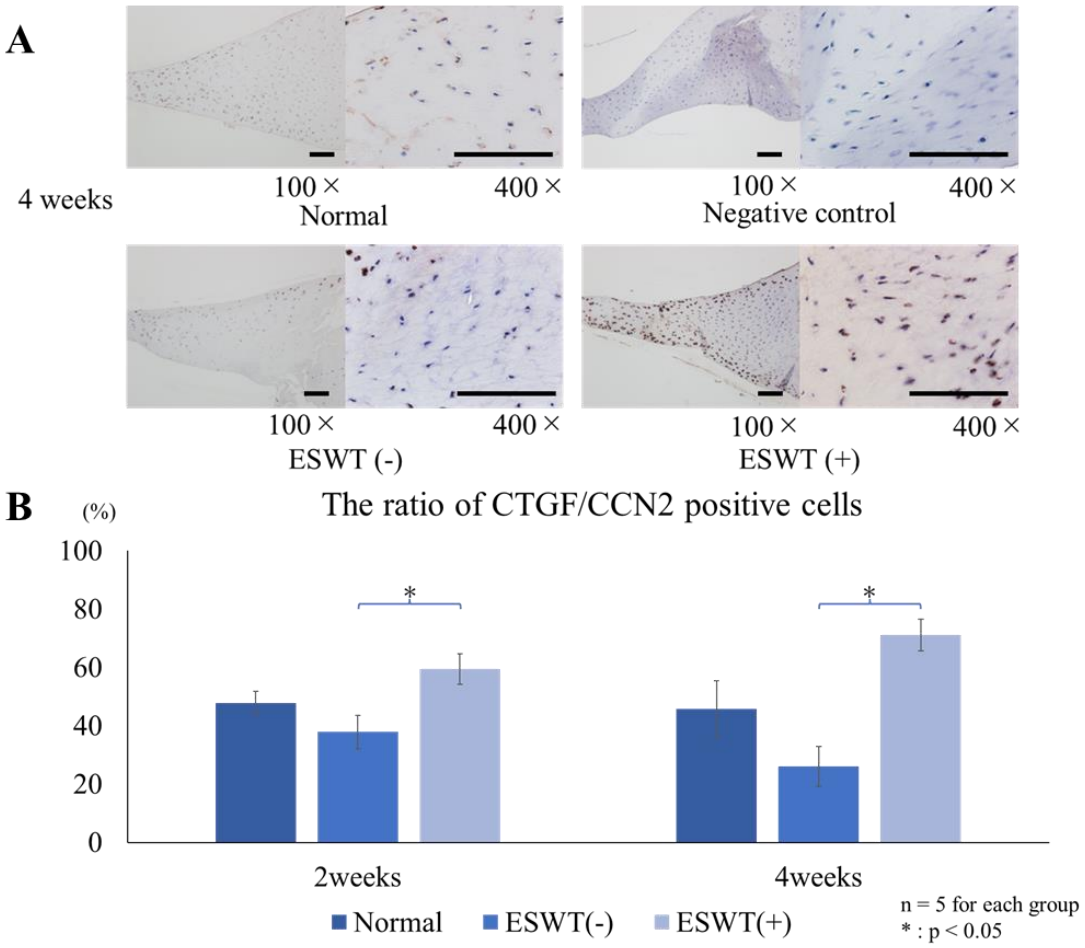


Figure 2. (A) Immunostaining with anti-Connective tissue growth factor/CCN family member 2 (CTGF/CCN2) antibody of the medial meniscus posterior segment in the normal, extracorporeal shockwave therapy (ESWT)–, and ESWT+ groups at 4 weeks after ESWT. (B) Comparison of the ratios of CTGF/CCN2-positive cells to all cells at 2 and 4 weeks after ESWT. Data are expressed as the mean ± standard deviation. P values were obtained with a Kruskal–Wallis test and post hoc test with a Dunn-Bonferroni analysis. *P<0.05 (n=5 per group). Bar=100μm

Immunostaining of the medial meniscus posterior segment with an anti-Ki67 antibody was performed in the normal, ESWT–, and ESWT+ groups at 2 and 4 weeks after ESWT (Figure 3A). The ratio of Ki67-positive cells to all cells is shown in Figure 3B. This ratio did not change at any of the observation time points in the normal group (55.4% and 53.6%, respectively). The positive cell ratio in the ESWT+ group was significantly increased at 2 weeks (p=0.022). This ratio was significantly higher in the ESWT+ group than in the ESWT– group at 2 weeks (p=0.027) and 4 weeks (p=0.002).

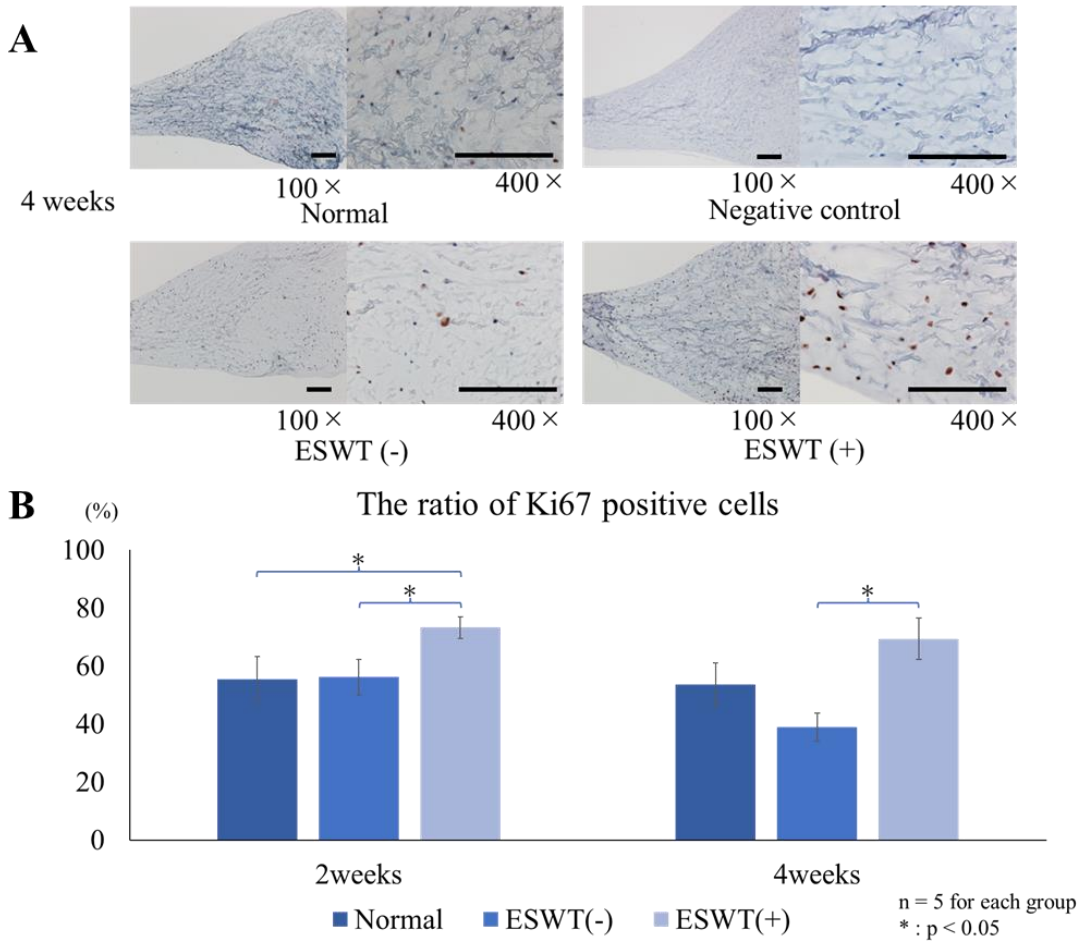


Figure 3. (A) Immunostaining with anti-Ki67 antibody of the medial meniscus posterior

segment in the normal, extracorporeal shockwave therapy (ESWT)–, and ESWT+ groups at 4 weeks after ESWT. (B) Comparison of the ratios of Ki67-positive cells to all cells at 2 and 4 weeks after ESWT. Data are expressed as the mean ± standard deviation. P values were obtained with a Kruskal–Wallis test and post hoc test with a Dunn-Bonferroni analysis. *P<0.05 (n=5 per group). Bar=100μm

The expression of collagen type 2 in the ESWT+ group at 4 weeks was higher than that in the ESWT– group in all areas of the meniscus (Figure 4).

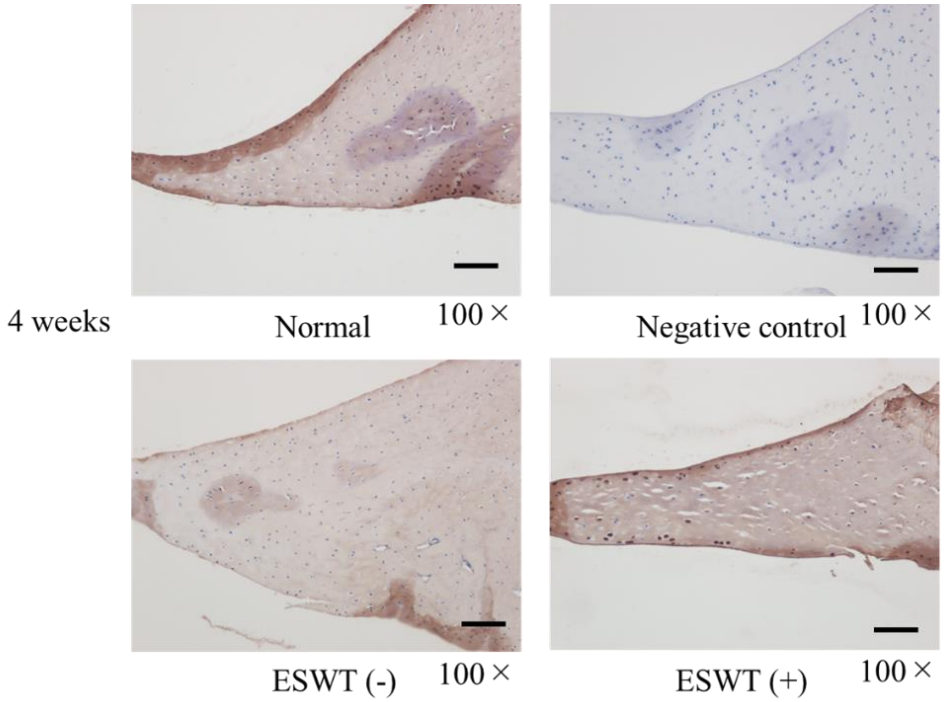


Figure 4. Immunostaining with anti-collagen type 2 antibody of the medial meniscus posterior segment in the normal, extracorporeal shockwave therapy (ESWT)–, and ESWT+ groups at 4 weeks after ESWT. Bar=100μm

272

273 **Gene Expression Analysis**

274 The mRNA expression of CTGF/CCN2, SOX9, VEGF-α, Acan, Col1a2, and Col2a1

275 was examined at 24 h and 4 weeks after ESWT by real-time PCR (Figure 5).

276 The mRNA expression at 24 h after ESWT may reflect the early effects of ESWT on the

277 degenerated medial meniscus. The mRNA expression of CTGF/CCN2 was significantly

278 higher in the normal group than in the ESWT– group at 24 h after ESWT (p=0.003)

279 (Figure 5A). Additionally, there was no significant difference between the ESWT+ and

280 normal groups at the same time point (Figure 5A). The mRNA expression of Col2a1

281 was significantly higher in the normal group than in the ESWT– group at 24 h after

282 ESWT (p=0.049). Additionally, there was no significant difference between the ESWT+

283 and ESWT– groups at the same time point (Figure 5A).

284 The mRNA expression of CTGF/CCN2 in the normal group was significantly

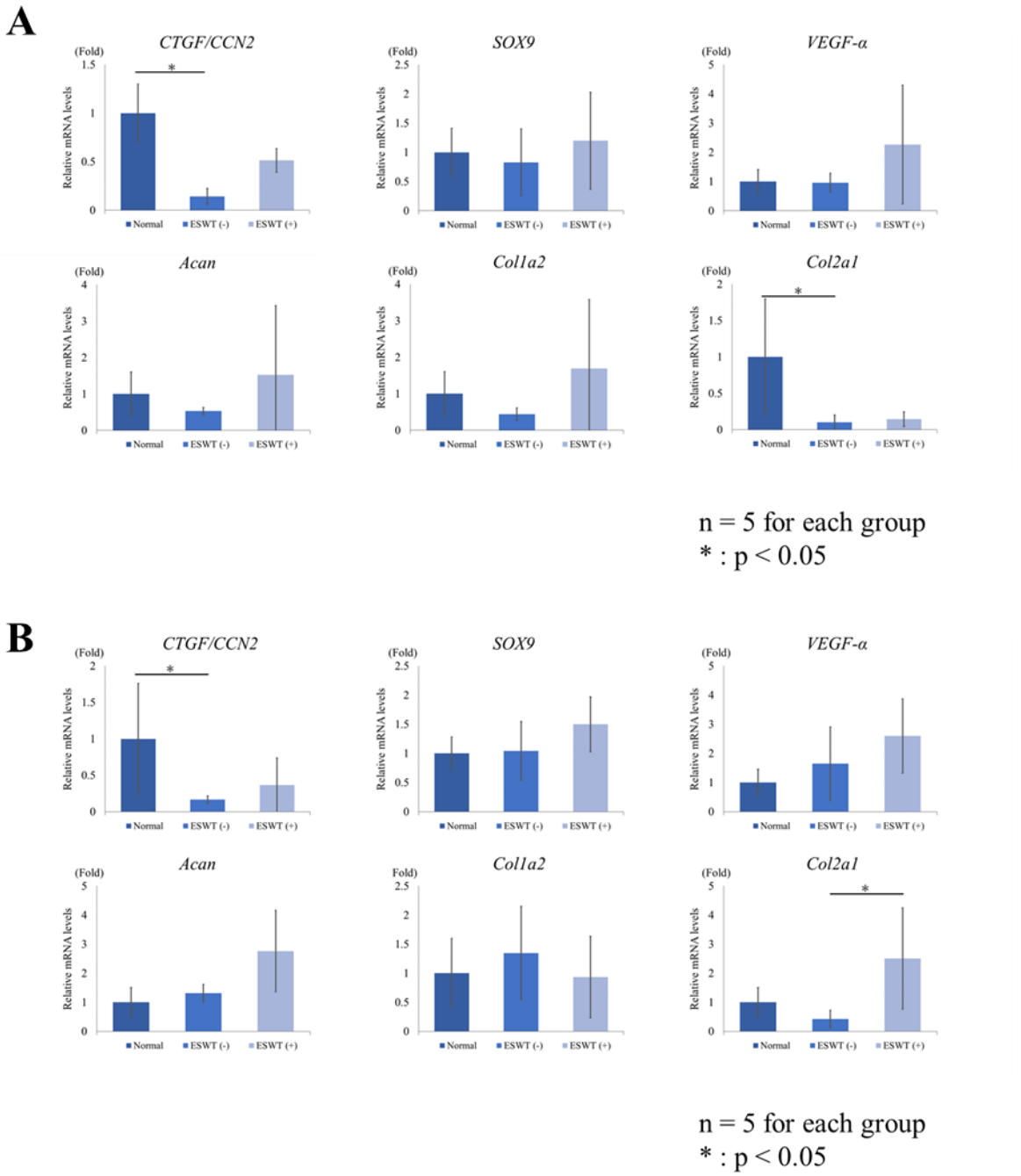
285 higher than that in the ESWT– group at 4 weeks after ESWT (Figure 5 B). The mRNA

286 expression of Col2a1 in the ESWT+ group was significantly higher than that in the

287 ESWT– group at 4 weeks after ESWT (p=0.009) (Figure 5B). In the three groups, the

288 mRNA expression of SOX9, VEGF-α, Acan, and Col1a2 did not differ significantly at

289 any time point (Figure 5).



290

291 **Figure 5.** The mRNA levels of Connective tissue growth factor/CCN family member 2

292 (CTGF/CCN2), SRY (sex determining region Y)-box 9 (SOX9), vascular endothelial

293 growth factor α (VEGF-α), aggrecan (Acan), collagen type 1 alpha 2 (Col1a2), and

294 collagen type 2 alpha 1 (Col2a1) were evaluated by real-time polymerase chain reaction

295 at (A) 24 h and (B) 4 weeks after extracorporeal shockwave therapy (ESWT). The
296 expression of these genes was normalized to the expression of glyceraldehyde 3-
297 phosphate dehydrogenase. The ordinate indicates the relative ratio to the normal group.
298 Data are expressed as the mean ± standard deviation. P values were obtained with a
299 Kruskal–Wallis test and post hoc test with a Dunn-Bonferroni analysis. *P<0.05 (n=5
300 per group).
301
302

Discussion

The critical finding of the present study is that ESWT can delay degenerative changes in the ACLT model rat meniscus. ESWT significantly suppressed degenerative changes in the meniscus, as evaluated by the histological scoring system. This effect may be due to the upregulation of CTGF/CCN2 and Col2a1. The moderate stimulation of meniscus cells by ESWT might have activated cartilage repair factors in meniscus cells. As a result, ESWT may promote intrinsic meniscal repair and suppress degenerative changes.

Degenerative change in the meniscus after ACLT

In this study, the degeneration of the medial meniscus was more evident at 4 weeks post-ACLT in comparison to the normal meniscus. In addition, meniscal histopathological scores increased over time. The ACLT model of knee osteoarthritis has long been used in animal studies, including rat studies. ACLT does not directly damage the meniscus, but degenerative histological changes in the medial meniscus are observed in the subsequent follow-up period[8,15]. Anterior and rotational instability of the knee joint after ACL injury increases the mechanical load on the medial meniscus, resulting in meniscus damage[10,24,27]. Similarly, in menisci from rat knees, degenerative changes in the posterior medial meniscus progress after ACLT alone. Specifically,

321 degenerative meniscus scoring was significantly higher in the posterior medial meniscus
322 at 4 weeks after ACLT, and degenerative histological changes were observed as a
323 result[15]. In addition, in a study using a rat model of knee osteoarthritis induced by a
324 single injury to the ACL, slight fibrillatory changes on the surface of the medial
325 meniscus were observed three weeks postoperatively[3]. Therefore, our results suggest
326 that ACLT increased the mechanical load on the medial meniscus, which could not be
327 completely repaired by the potential healing ability of the meniscus, and that the
328 degeneration progressed over time.

329 In the present study, the mRNA expression of Col2a1 and CTGF/CCN2 was
330 decreased in the posterior medial meniscus during degeneration after ACLT. Previous
331 studies reported that a decrease in collagen type 2 has been identified in the process of
332 cartilage degeneration after an anterior cruciate ligament injury, and this condition is
333 known to induce osteoarthritis of the knee[20]. Collagen type 2 plays a critical role in the
334 meniscus and is downregulated in the degenerated meniscus in a mouse with ACLT-
335 induced meniscus degeneration[35]. In chondrocytes, CTGF/CCN2 is involved in the
336 regulation of collagen type 2, expression of aggrecan, and cell proliferation, and its
337 expression is upregulated by moderate mechanical loading[7,9,21]. However, the
338 expression of CTGF/CCN2 is downregulated in chondrocytes and fibroblasts under

339 excessive constant cyclic loading[14,18]. Furthermore, the expression of CTGF/CCN2 is
340 suppressed in osteoarthritis-induced meniscus cells[26]. The expression of CTGF/CCN2
341 and Col2a1 in the medial meniscus after ACLT in this study may have been suppressed
342 by the excessive mechanical loading on the meniscus provided by anterior tibial
343 instability. The decreased expression of Col2a1 during the process of medial meniscus
344 degeneration in this study may indicate a catabolic effect, similar to that of articular
345 cartilage degeneration in knee osteoarthritis.
346
347 **The effects of ESWT on the degenerated meniscus**
348 In this study, ESWT suppressed the progression of ACLT-induced meniscus
349 degeneration. The meniscal degeneration score in the ESWT+ group was significantly
350 lower than that in the ESWT– group at 4 weeks and 12 weeks. In addition, in the
351 present study, we observed upregulation of CTGF/CCN2 at 24 h after ESWT and
352 increased the CTGF/CCN2-positive cell ratio at 2 and 4 weeks after ESWT. A previous
353 study revealed that ESWT promotes the healing of meniscus tears by stimulating the
354 proliferation of meniscus cells and the expression of genes, including CTGF/CCN2,
355 SOX9, aggrecan, and Col2a1[11]. Meniscus grafts with CTGF/CCN2 and TGF-β
356 regenerated the knee meniscus, enabled functional knee recovery, and synthesized zone-

357 specific collagen types 1 and 2[17]. The overexpression of CTGF/CCN2 in articular
358 cartilage alleviates degenerative changes by promoting the synthesis proteoglycan and
359 the proliferation chondrocytes[13]. In addition, ESWT has a chondroprotective effect and
360 inhibits the progression of knee osteoarthritis induced by ACLT in rats[30,34]. Our results
361 may demonstrate that CTGF/CCN2 was activated in the degenerated meniscus similarly
362 to in the process of promoting the healing of meniscus injury by ESWT effects that
363 activate intrinsic repair factors. These effects might be factors that suppress the
364 progression of histological degenerative changes.

365 In this study, the expression of Col2a1 was upregulated after ESWT for 4 weeks.
366 In addition, immunostaining of anti-collagen type 2 showed stronger stainability in the
367 ESWT+ group than in the ESWT– group. In this study, meniscus cell proliferation was
368 observed after ESWT. Collagen type 2 forms a fibrous network in the tissue and
369 provides tensile strength to the meniscus[11,16]. In a study using cultured tenocytes, ESWT
370 promoted cell proliferation and collagen synthesis[28]. The protective effect of ESWT on
371 articular cartilage is derived from activating chondrocytes and increasing the expression
372 of collagen type 2[30]. In addition, even when ESWT was applied after the onset of knee
373 osteoarthritis, a delay in osteoarthritic changes was demonstrated[34]. Another study
374 reported that ESWT increases the expression of SOX9 in the meniscus[11]; however, in

the present study, we did not observe a significant increase in the SOX9 expression after ESWT. This may be influenced by differences in the response of the meniscus cells during the injury repair process and the process of degeneration. In the present study, ESWT increased the number of CTGF/CCN2-positive cells, promoted cell proliferation, and suppressed degenerative changes in the degenerated meniscus. Our results demonstrated that ESWT has protective effects on the degenerated meniscus that are similar to the protective effects on articular cartilage. The onset of this effect may be related to the activation of endogenous cartilage repair factors in meniscus cells, but detailed research is needed to clarify this process.

The present study was associated with some limitations. First, the present study used a rat model. The load on the knee joints is 25% in rats, whereas it is 50% in humans. In addition, because of the difference in anatomy and composition (e,g., menisci ossification) between the rat and human meniscus, the effects of ESWT on humans and large animals may be different. Second, the present study did not reveal what happened after 12 weeks post-ESWT. To investigate the long-term effects of ESWT on the degenerated meniscus, a longer follow-up period is needed. Third, we did not evaluate the articular cartilage. The effects of ESWT on degenerative changes

393 in the cartilage were not clarified in this study. However, several studies[29,32,33] have
394 revealed these effects, so we focused on the effects on the meniscus. Fourth, the
395 present study only analyzed the effect of a single ESWT session; thus, the optimal
396 number of sessions for the therapeutic effect is unclear. Fifth, the degenerative changes
397 of this study reflect post-traumatic osteoarthritis induced by ACLT and not primary
398 osteoarthritis. However, ACLT does not directly damage the meniscus and
399 degenerative changes can be observed. Sixth, there was no sham surgery group in this
400 study. We tried to focus on the effects of ESWT. Seventh, the mechanical properties
401 and inflammatory effects were not evaluated in this study. Eighth, not all cells were
402 counted when determining the number of meniscal cells, rather the percentage of
403 meniscal cells in the frame was used. Ninth, all of the rats included in this study were
404 male.
405
406 **Conclusion**
407 ESWT suppressed the progression of meniscus degeneration in a rat model of meniscus
408 degeneration after ACLT. Immunostaining and real-time PCR revealed that ESWT
409 increased the expression of CTGF/CCN2 in the degenerated meniscus. Additionally,
410 ESWT increased the number of Ki67-positive cells. ESWT promotes the expression of

411 mRNA in collagen type 2. These results indicated that ESWT can be an option for the

412 treatment of degenerated meniscal tissue in a rat model.

413

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