Extracorporeal shock wave promotes activation of anterior cruciate ligament remnant cells and their paracrine regulation of bone marrow stromal cells’ proliferation, migration, collagen synthesis, and differentiation

Aims

Proliferation, migration, and differentiation of anterior cruciate ligament (ACL) remnant and surrounding cells are fundamental processes for ACL reconstruction; however, the interaction between ACL remnant and surrounding cells is unclear. We hypothesized that ACL remnant cells preserve the capability to regulate the surrounding cells’ activity, collagen gene expression, and tenogenic differentiation. Moreover, extracorporeal shock wave (ESW) would not only promote activity of ACL remnant cells, but also enhance their paracrine regulation of surrounding cells.

Methods

Cell viability, proliferation, migration, and expression levels of Collagen-I (COL-I) A1, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) were compared between ACL remnant cells untreated and treated with ESW (0.15 mJ/mm², 1,000 impulses, 4 Hz). To evaluate the subsequent effects on the surrounding cells, bone marrow stromal cells (BMSCs)’ viability, proliferation, migration, and levels of Type I Collagen, Type III Collagen, and tenogenic gene (Scx, TNC) expression were investigated using coculture system.

Results

ESW-treated ACL remnant cells presented higher cell viability, proliferation, migration, and increased expression of COL-I A1, TGF-β, and VEGF. BMSC proliferation and migration rate significantly increased after coculture with ACL remnant cells with and without ESW stimulation compared to the BMSCs alone group. Furthermore, ESW significantly enhanced ACL remnant cells’ capability to upregulate the collagen gene expression and tenogenic differentiation of BMSCs, without affecting cell viability, TGF-β, and VEGF expression.

Conclusion

ACL remnant cells modulated activity and differentiation of surrounding cells. The results indicated that ESW enhanced ACL remnant cells viability, proliferation, migration, and expression of collagen, TGF-β, VEGF, and paracrine regulation of BMSC proliferation, migration, collagen expression, and tenogenesis.

Cite this article: Bone Joint Res 2020;9(8):457–467.

Keywords: Anterior cruciate ligament, Extracorporeal shock wave, ACL reconstruction, Graft maturation

Article focus

The interaction between anterior cruciate ligament (ACL) remnant and its surrounding cells after ACL reconstruction with remnant preservation.
The effects of extracorporeal shock wave (ESW) on activity of the ACL remnant cells and paracrine regulation of surrounding cells.

**Key messages**

- After ACL reconstruction with remnant preservation, the ACL remnant regulated bone marrow stromal cells (BMSCs) proliferation, migration, collagen gene expression, and tenogenesis potential to help graft maturation.
- ESW stimulated the viability, proliferation, migration, and expression of collagen synthesis, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) of ACL remnant cells.
- Overall, ESW treatment not only stimulated the activity of ACL remnant cells, but also significantly enhanced its regulatory capability to the surrounding cells, which would help in graft maturation.

**Strengths and limitations**

- The first study to confirm the interaction between ACL remnant and its surrounding cells and the effects of ESW on ACL remnant cells and their regulatory capability to the surrounding cells.
- We investigated the effects of ESW on ACL remnant cells in vitro but did not validate these findings using animal studies.
- The optimal dose and frequency of ESW to treat ACL remnant cells and promote tissue regeneration was not investigated.

**Introduction**

Knee injuries, especially those involving the anterior cruciate ligament (ACL), are common sports injuries. Due to its intra-articular location, limited blood supply, and intrinsic cell loss, the injured ACL heals poorly. Surgical ACL reconstruction recovers joint function and athletic ability in clinical practice. The major concerns of implanted graft after ACL reconstruction are osseous integration at the graft-tunnel interface and remodeling of the intra-articular graft (ligamentization). After implantation, the graft necrosis leads to secretion of growth factors and cytokines that direct cell migration from the graft periphery (bone marrow, synovium, and remnant of the native ACL) to the injury site. These migrated cells further proliferate and produce extracellular matrix, and proceed tenogenic differentiation to promote ligament healing under growth factor stimulation. Hence, insights into the regulation of ACL remnant tissue and cells and surrounding cells in terms of proliferation, migration, collagen synthesis, secretion of growth factors, and differentiation is important to accelerate early graft healing and prevent graft rupture after ACL reconstruction.

The remnant preservation technique for ACL reconstruction has been proposed to improve functional result and augment graft incorporation by enhancing cell proliferation, revascularization, and regeneration of proprioceptive organs. However, the clinical value and result after remnant preservation in ACL reconstruction is still debated. The ACL remnant was demonstrated to contain stem cells and present the capability to augment the healing of tendon to bone tunnel in in vitro studies. During ACL reconstruction, ACL remnant surrounded the implanted graft and contacted the surrounding tissue and cells (i.e. bone marrow stromal cells (BMSCs) released from the drilled bone tunnel that covered ACL remnant and implanted graft; Figure 1 middle and right images). However, the actual role of ACL remnant cells in graft regeneration and their interaction with surrounding cells is not clear.

Extracorporeal shock wave (ESW), a sound wave characterized with fast pressure rise and high peak-pressure in a short lifecycle, has been applied in treating multiple...
orthopaedic disorders. The possible mechanisms of ESW treatment include mechanical force stimulation, signalling pathway transduction, biomolecule secretion, ion channel alteration, and cytokine and growth factor release. ESW treatment potentiates soft tissue regeneration. Studies have shown that ESW treatment activates proliferation, migration, and gene expression in different cells. ESW has been proposed as a non-invasive treatment that potentiates graft healing after ACL reconstruction surgery. Wang et al treated rabbits with 500 impulses of shock waves at 14 kV after ACL reconstruction using long digital extensor tendons, and demonstrated a significant improvement in healing at the grafted tendon to bone tunnel interface. While these results are promising, the effects of ESW on the biochemical processes of ACL remnant and surrounding cells must be elucidated in vitro to better inform subsequent clinical studies evaluating ESW to improve the graft maturation after ACL reconstruction.

In this study, we hypothesized that ACL remnant cells would modulate the behaviour of surrounding cells including their exhibited proliferation, migration, and collagen formation. We also hypothesized that ESW treatment would not only activate the ACL remnant cells, but also strengthen their paracrine and downstream capability to modulate BMSC activity and differentiation.

**Methods**

**Cell isolation.** ACL remnant tissues were obtained from eight patients (five males and three females; mean age 23.5 years (SD 3.46); mean injury time 3.3 months (SD 1.6)) who underwent ACL reconstruction surgery. These ACL remnant tissues were harvested by arthroscopic punch during operation (Figure 1 left image). This study procedure was approved by the Institutional Review Board (KMUHIRB-F(I)-20160112). After removing the epitendon and peritendon sheaths, the remnant tissues were minced into pieces 1 mm to 2 mm thick and washed with phosphate-buffered saline (PBS). Tissues were spun down at 1,000 rpm, 25 °C for five minutes, collected, and cultured in low-glucose Dulbecco’s modified Eagle’s medium (Gibco DMEM; Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% foetal bovine serum (Gibco FBS; Thermo Fisher Scientific) and 1% antibiotics (penicillin/streptomycin; Gibco; Thermo Fisher Scientific) at 37°C in a 5% CO₂ incubator. Cells that migrated from the remnant tissue without collagenase digestion after seven to ten days were designated the P0 passage and subcultured with 0.25% trypsin (Gibco; Thermo Fisher Scientific) until 80% confluence was reached. The third passage ACL remnant cells were used for this study.

Rabbit BMSCs were isolated from 8 ml of bone marrow, previously aspirated from iliac crest, and cultured. The animal experiment protocol was approved by the Institutional Animal Care and Use Committees (IACUC) (KMU-105263). The third passage BMSCs were used for this study.

**ESW treatment protocol.** ESW was administered in focused mode with an electromagnetic shock wave generator (Duolith SD1; Storz Medical AG, Trägerwilen, Switzerland). Third-passage remnant cells at a density of 1 × 10⁶ cells/ml were placed in a cryogenic vial and subjected to 1,000 shock wave impulses at 0.15 mJ/mm². The control group did not receive ESW treatment.

**Coculture of ACL remnant cells with BMSCs.** To investigate the regulatory capability of ACL remnant cells on other cells, we cocultured the human ACL remnant cells with rabbit BMSCs using a non-contact method with the transwell system (4 µm pores). BMSCs (1 × 10⁵) were plated on the floor of the culture plate (lower well) with MEM α (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) incubated at 37°C and 5% CO₂. After 24 hours the culture medium was discarded, and the non-ESW-treated ACL remnant cells (ACL-ESW coculture group) or the ACL remnant cells that were treated with ESW (1,000 shock wave impulses at 0.15 mJ/mm²; ACL+ESW coculture group) were seeded on the transwell insert (upper well) with medium (low-glucose DMEM, serum-free, 1% antibiotics (penicillin/streptomycin); all from Gibco; Thermo Fisher Scientific). As control group, 1 × 10⁴ BMSCs were cultured in monolayers without coculture with ACL remnant cells. After seven days, the BMSCs in each group were detached with 0.25% trypsin and subcultured to be used in other experiments.

**Cell viability.** Cell viability was determined for ESW-treated and untreated ACL remnant cells and for all BMSCs in the coculture experiments (control group, ACL-ESW coculture group, and ACL+ESW coculture group) by the MTT assay following the manufacturer’s instructions. The third passages of ACL remnant cells and BMSCs were used in this study. These cells were cultured in 96-well plates at a density of 5 × 10⁴ cells/well containing 200 µl medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Absorbance was measured at 570 nm using a Bio-Rad Microplate Manager Benchmark Plus Reader (Bio-Rad Laboratories, Hercules, California, USA). The results were compared between the ESW-treated and untreated ACL remnant cells, and between the BMSCs in the control group, ACL-ESW coculture group, and ACL+ESW coculture group.

**Cell proliferation- EdU assay.** Cell proliferative ratio was analyzed with a Click-iT EdU assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The third passages of ESW-treated and untreated ACL remnant cells and BMSCs in different coculture groups were cultured in 12-well plates at a density of 2 × 10⁴ cells/well containing 2 ml medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Cell slides were prepared with mounting medium and counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, USA) for ten minutes.
Slides were viewed under fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). The cell proliferation rate was calculated by ImageJ (64-bit Java v. 1.6.0_24; National Institutes of Health (NIH), Bethesda, Maryland, USA) using five randomized areas per sample that was captured by computerized stage set and analyzed by scientists who were blinded to the study groups.

RNA isolation and real-time polymerase chain reaction. The third passages of ESW-treated and untreated ACL remnant cells and BMSCs in different coculture groups were cultured in six-well plates at a density of $2 \times 10^5$ cells/well containing 3 ml medium (MEM supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Total RNA was extracted with RNeasy reagent (MRC Inc, Cincinnati, Ohio, USA). Then, 2 μg of purified total RNA was reverse-transcribed using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Real-time PCR was carried out using the SYBR Green PCR Master Mix (Thermo Fisher Scientific) and was processed on ABI 7900 real time PCR instrument. Each reaction (20 μl) was run in triplicate and contained 1 μl of complementary DNA (cDNA) template along with the relevant primers (Table I). Threshold cycle (Ct) for all tested genes were normalized to that of GAPDH (ΔCt). Each experimental sample was referred to its control (ΔΔCt). Fold change values were expressed as $2^{-\Delta \Delta \text{Ct}}$.

Table I. The primers for complementary DNA (cDNA) sequences.

| Gene name | Amplicon size, bp | Primer sequence | Accession no. |
|-----------|-------------------|-----------------|--------------|
| Hu-Ki67   | 78                | 5'-GAGGTGTGGCAGAAAATCCAAA-3' 5'-CTGTCCCTATGACTCTGCTTG-3' | NM_001145966.2 |
| Hu-GAPDH | 576               | 5'-CCATCATCATCTCCACGAGG-3' 5'-TGGTCTGACACCTTTCCTTG-3' | NM_001257999.3 |
| Rb-COL-1 | 73                | 5'-TTCTCGAGGCCCTCAGA-3' 5'-TGGTCCAGAGATGATACGTC-3' | NM_001195668.1 |
| Rb-COL-3 | 92                | 5'-CCCTGACGGGAGGCAAA-3' 5'-TGGTCCAGAGATGATACGTC-3' | XM_002712333.3 |
| Rb-TGFβ  | 140               | 5'-ACTGCGGACAGGAGGACAC-3' 5'-CAGGCCCCACCGGGATTC-3' | XM_008210800.2 |
| Rb-VEGF  | 122               | 5'-ATCACCGGATCAAACCTCTC-3' 5'-CAGGCCCCACCGGGATTC-3' | XM_020912278.1 |
| Rb-Ki67  | 232               | 5'-GTCACGGGAGAGGACAC-3' 5'-CAGGCCCCACCGGGATTC-3' | XM_008210804.2 |
| Rb-SCX   | 165               | 5'-CGACCGCAGAGGACGACAC-3' 5'-CAGGCCCCACCGGGATTC-3' | BK0002280 |
| Rb-TNC   | 78                | 5'-CAGAAAGCTTGCCGATTGG-3' 5'-CACTCTCTCCTGCTTACG-3' | XM_017350093 |
| Rb-GAPDH | 103               | 5'-AGTGCACCCCACCTCCAC-3' 5'-TGCTGTGACAAATCCGGTG-3' | NM_001082253 |

Hu: human; Rb: rabbit; COL-1: Collagen-I; COL-3: Collagen-III; TGFβ: transforming growth factor beta 1; VEGF: vascular endothelial growth factor; Ki67: marker of proliferation Ki-67; SCX: scleraxis; TNC: Tenascin D; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

ImageJ was used to calculate the relative changes in cell migration rate.

Transwell migration assay. An in vitro cell migration assay was performed in a 6.5 mm Transwell chamber with an 8 μm pore diameter (EMD Millipore, Billerica, Massachusetts, USA). The third passages of ACL remnant cells with or without ESW treatment and BMSCs in different coculture groups ($3 \times 10^4$ cells/well) in serum-free medium were seeded on the upper chamber compartment for the migration assay. MEM α supplemented with 10% FBS and 1% penicillin/streptomycin was added to the lower chamber compartment as a chemoattractant. After incubation for 20 hours at 37°C, cells that migrated to the lower membrane surface were fixed with 4% paraformaldehyde (PFA) for ten minutes, followed by staining with 0.5% crystal violet for 20 minutes. Migrated cells were counted in each chamber under a microscope and the numbers were normalized to those of the control cells. The relative cell migration ability was calculated as the ratio of migrating treated cells to the control cells and designated as the migration rate (% of the control). Each reaction was performed in triplicate and the mean was obtained from those results.

Immunofluorescence staining. ACL remnant cells with or without ESW treatment and BMSCs in different coculture groups were seeded in 16 mm cover glasses at a density of $2 \times 10^4$ cells/well containing 2 ml medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) and incubated at 37°C for 48 hours. The cells were then fixed with 4% PFA for 15 minutes and 1% Triton-X-100 for one minute, and then preincubated with 100 μl blocking solution (1% FBS in PBS) for 20 minutes. Primary antibodies against Type I Collagen (Sigma-Aldrich), Type III Collagen (Arigo Biolaboratories, Hsinchu, Taiwan), transforming growth factor beta (TGF-β, Sigma-Aldrich), and VEGF proteins (Arigo Biolaboratories) were used to stain the cells overnight at 4°C. The cells were then stained with fluorescence secondary antibodies; Donkey anti-Goat immunoglobulin G (IgG) (H + L)-FAM (1:250; Leadgene Biomedical, Tainan, Taiwan) for Type
EXTRACORPOREAL SHOCK WAVE PROMOTES ACTIVATION OF ANTERIOR CRUCIATE LIGAMENT REMNANT CELLS

I Collagen and TGF-β in ACL remnant cells and Type I and Type III Collagen in BMSCs; Goat anti-Mouse IgG (H + L)-TAMRA (1:250; Leadgene Biomedical) for TGF-β in BMSCs and VEGF in both ACL remnant cells and BMSCs for one hour and rinsed thrice with PBS. The slides were counterstained with 1 μg/ml Hoechst solution (Sigma-Aldrich) for ACL remnant cells and DAPI for BMSCs for ten minutes, rinsed thrice with PBS, and mounted before observation under a confocal microscope. ImageJ was used to quantify fluorescence intensity in five random fields of six samples that was captured by computerized stage set and analyzed by scientists who were blinded to the study groups. The results of fluorescence intensity in ACL remnant cells after ESW treatment were shown in fold change compared with those of ACL remnant cells without ESW treatment.

Statistical analysis. The differences between ESW-treated and untreated ACL remnant cells were analyzed by the paired t-test. In the coculture experiment, we compared the results among the control, ACL-ESW coculture, and ACL+ESW coculture groups using one-way analysis of variance (ANOVA) with Tukey’s post hoc test. All data are presented as mean (SD). p < 0.05 was considered statistically significant. All statistical analysis was performed using SPSS software version 20 (IBM, Armonk, New York, USA).

Results
ESW treatment increased ACL remnant cell viability, proliferation, and migration. The ACL remnant cell viability significantly increased within 72 hours of ESW treatment (Figure 2a). The proliferation rates markedly increased in ACL remnant cells after ESW treatment in both EdU assay and Ki67 gene expression compared with that of untreated cells (Figure 2b). Furthermore, the ACL remnant cells treated with ESW more actively migrated into the scratched area (upper panel) or lower chamber compartment (lower panel) than the untreated cells (Figure 2c).

ESW treatment upregulated COL-I A1, TGF-β, and VEGF expression in ACL remnant cells. We conducted immunofluorescence staining to detect Collagen-I (COL-I) A1, TGF-β, and VEGF expression after ESW treatment. COL-I A1, TGF-β, and VEGF protein levels were all significantly upregulated in ESW-treated ACL remnant cells relative to those in the untreated cells (Figure 3).

BMSC proliferation and migration rate increased after coculture with ACL remnant cells with and without ESW stimulation. The cell viability of BMSCs did not reveal significant change between control, ACL-ESW coculture, and ACL+ESW coculture group (Figure 4a). BMSCs showed higher cell proliferation rate than control group after coculture with ACL remnant cells (in both ESW-treated and non-treated groups), according to EdU assay and Ki67 gene expression levels (Figure 4b). The scratch migration test revealed significantly higher BMSC migration rate after 12 or more hours of coculture with ACL remnant cells, and the BMSCs in the ACL+ESW coculture group showed highest migration rate among the three groups at all timepoints (Figure 4c upper panel). These results were consistent with the transwell migration study results (Figure 4c lower panel). In both the proliferation and migration studies, the ESW-treated ACL remnant cells presented a more profound effect on BMSC activity compared to non-ESW-treated ACL remnant cells.

ESW enhanced ACL remnant cells’ capability to upregulate BMSC collagen gene expression and tenogenic differentiation, without affecting TGF-β and VEGF expression. The BMSCs cocultured with ACL remnant cells showed significantly increased levels of Type I and Type III collagens (Figure 5) and tenogenic gene (Scx and TNC) expression.
Effects of extracorporeal shock wave (ESW) treatment on Collagen-I (COL-I) A1, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) expression in anterior cruciate ligament (ACL) remnant cells. Immunofluorescence imaging and results of mean fluorescence intensities (MFI) (n = 6) showed that COL-I A1 (upper panel; stained with FAM in green), TGF-β (middle panel; stained with FAM in green), and VEGF (lower panel; stained with TAMRA in red) protein expression levels in the ESW-treated ACL remnant cells were significantly higher than those in untreated cells. Cell nuclei were counterstained with Hoechst in blue. All images are shown under 200× magnification. Data are indicated as means (SD). Scale bar = 50 μm. *p < 0.01.

**Discussion**

Enhancing the proliferation, migration, and differentiation of ACL remnant and peripheral cells is important to improve graft maturation after ACL reconstruction with remnant preservation. This study demonstrated that ACL remnant cells exerted a paracrine effect to regulate proliferation, migration, collagen synthesis, and tenogenic differentiation of BMSCs. ESW treatment activated cell viability, proliferation, migration, and increased COL-I A1, TGF-β, and VEGF expression in ACL remnant cells compared to those of untreated cells, and their paracrine effect on BMSCs was further increased after ESW stimulation, compared to untreated ACL remnant cells. Therefore, ESW treatment could improve graft maturation after ACL reconstruction by both activating remnant cells and enhancing their paracrine effect to regulate the surrounding cells to proliferate, migrate, and synthesize collagen and differentiate.

Studies have reported that ACL remnant tissue contains both mesenchymal and vascular stem cells, which play important roles in injury repair. However, another study showed poor structure and low graft healing capacity in the presence of ACL remnant cells. Similarly,
Lu et al.

Effects of anterior cruciate ligament (ACL) remnant cells on bone marrow stromal cells (BMSCs)' viability, proliferation, and migration. a) No significant difference of BMSCs viability was found between the control group, ACL-extracorporeal shock wave (ESW) coculture group, and ACL+ESW coculture group (n = 6). b) BMSCs proliferation rate in ACL remnant cells coculture group was significantly higher than that in the control group. The ESW-treated ACL remnant cells coculture group showed a more pronounced effect than non-treated ACL remnant cells coculture group (n = 6). Scale bar = 50 μm. c) Significantly higher BMSCs migration rate was noted in the ACL coculture group compared to the control group. The cell migration rate of BMSCs in the ESW-treated ACL remnant cells coculture group was the highest among the three groups. (Scratch migration, n = 6; Insert migration, n = 4). Scale bar = 100 μm. All data are means (SD). *p < 0.05; †p < 0.01 between two comparison groups; ‡p < 0.05, ACL- ESW coculture group versus control group; §p < 0.05, ACL+ESW coculture group versus control group. Ctrl, control; mRNA, messenger RNA.
Effects of anterior cruciate ligament (ACL) remnant cells on Type I Collagen (Col-I), Type III Collagen (Col-III), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) expression in bone marrow stromal cells (BMSCs). After coculture with ACL remnant cells, BMSCs evidenced higher Type I and Type III Collagen gene expression in both immunofluorescence staining (both were stained with FAM in green) and reverse transcription polymerase chain reaction (RT-PCR) results. BMSCs presented the highest gene expression of Type I Collagen and Type III Collagen in the ACL+ESW coculture group. No significant difference in the expression of TGF-β and VEGF (both were stained with TAMRA in red) between the control and ACL coculture groups with and without ESW treatment was noted (n = 6). Cell nuclei were counterstained with DAPI in blue. All images are shown with 200× magnification. Scale bar = 50 μm. Data are indicated as means (SD). *p < 0.05; †p < 0.01. Ctrl, control; mRNA, messenger RNA.

ESW treatment was shown to stimulate target cell activity. However, the interaction between ESW-treated cells and other cells has not been well investigated. In this study, the ESW treatment activated the ACL remnant cell viability, migration, collagen synthesis, and TGF-β and VEGF expression. Moreover, coculture with ESW-treated ACL remnant cells more profoundly regulated BMSC proliferation, migration, expression of collagen, and tenogenic differentiation compared to non ESW-treated ACL remnant cells and control group. The results indicated that ESW may not only activate the target cell directly, but also surrounding cells by promoting secretion of factors that regulate the activity and differentiation of the surrounding cells in a paracrine fashion. However, BMSC viability and secretion of growth factors did not improve after coculture with ESW-treated ACL remnant cells; consequently no significant difference to the results was obtained with non ESW-treated ACL remnant cells.
EXTRACORPOREAL SHOCK WAVE PROMOTES ACTIVATION OF ANTERIOR CRUCIATE LIGAMENT REMNANT CELLS

Fig. 6

Effects of anterior cruciate ligament (ACL) remnant cells on bone marrow stromal cells (BMSCs) tenogenic differentiation (Scx, TNC). After coculture with ACL remnant cells, BMSCs revealed higher Scx and TNC gene expression in reverse transcription polymerase chain reaction (RT-PCR) results. BMSCs cocultured with extracorporeal shock wave (ESW)-treated ACL remnant cells presented the highest Scx and TNC gene expression (n = 6). Data are indicated as means (SD). \(*p < 0.01\). Ctrl, control.

coculture and control coculture groups. These findings showed that ESW treatment could enhance native capability with inability to create new function of the target cell to regulate surrounding cells.

In clinical practice, ESW would not only be delivered to ACL remnant, but also to other tissues in the reconstruction area (e.g. tendon graft, bone marrow, synovium). Vetrano et al\(^{31}\) investigated the effect of ESW on tenocyte in semitendinosus tendon harvested during graft preparation in ACL reconstruction. Their result showed that ESW promotes the proliferation and collagen synthesis in tenocytes of the semitendinosus tendon. Suhr et al\(^{55}\) reported that ESW application increases human BMSC growth rate, proliferation, and migration and reduces the apoptosis rate. Their experimental result also showed that ESW could induce the remodelling of actin cytoskeleton and maintain the differentiation potentials in BMSCs after ESW treatment. Wang et al\(^{34}\) first investigated the effect of ESW in rabbit ACL reconstruction model; their result showed that ESW treatment significantly enhanced the interfacial healing between graft tendon and bone tunnel from the histomorphological and tensile strength test. Further, Wang et al\(^{33}\) applied the ESW treatment (1,500 impulses at 20 kV) delivery to tibial tunnel in patients who received remnant preservation ACL reconstruction surgery immediately during the same anaesthesia. At two years follow-up, the Lysholm score was significantly improved and tibial tunnel enlargement was decreased in the ACL reconstruction patients who received ESW compared to that in the no-ESW treatment patients. The current study is the first to hypothesize and demonstrate that ESW could enhance the activity of ACL remnant cells and its regulatory effect on BMSCs that might benefit in graft maturation. Nevertheless, the interaction between ACL remnant, graft tenocytes, BMSCs, and other tissue under ESW stimulation requires further investigation to clarify the effect of ESW on graft maturation in ACL reconstruction.

This study had some limitations. First, we investigated the effects of ESW on ACL remnant cells in vitro, but did not validate these findings with in vivo animal studies. Second, we acknowledge that the indirect coculture of human ACL remnant cells and rabbit BMSCs to simply observe the regulatory effect of ACL remnant cells on surrounding cells is a limitation. To understand the clinical situation in ACL reconstruction, a study using direct contact coculture of ACL remnant cells and surrounding cells from the same species will be needed. Third, we did not elucidate the clinical effects and precise mechanism of ESW treatment on ACL remnant tissue. Prior to the clinical application of ESW, its optimal dose and frequency to treat ACL remnant cells and promote tissue regeneration remain to be determined. Several studies reported dose-dependent influence of ESW on various cell activities and injured tissue healing.\(^{56-58}\) To the best of our knowledge, no study to date has validated the optimal ESW dose for
ACL remnant cells. Future experiments should be directed toward performing in vitro cell studies and ACL reconstruction with remnant preservation in animal models to confirm the efficacy and safety of ESW application before proceeding with clinical trials in human volunteers.

In conclusion, our study revealed a few novel findings. We demonstrated that ACL remnant cells regulated the activities of surrounding cells by increasing their proliferation, migration, collagen synthesis, and differentiation. The ESW treatment not only activated the ACL remnant cells’ viability, migration, collagen synthesis, and secretion of TGF-β and VEGF, but also significantly enhanced their paracrine capabilities to regulate the surrounding cells, thereby enhancing graft healing. These results support the evidence of application of ESW in ACL reconstruction with remnant preservation to enhance graft maturation.

References

1. Bradley JP, Klimkiewicz JJ, Rydel MJ, Powell JW. Anterior cruciate ligament injuries in the National Football League: epidemiology and current treatment trends among team physicians. Arthroscopy. 2002;18(5):502–509.

2. Gianotti S, Marshall SW, Hume PA, Bunt L. Incidence of anterior cruciate ligament injury and other knee ligament injuries: a national population-based study. J Sci Med Sport. 2009;12(6):622–627.

3. Sanders TL, Maradit Kremers H, Bryan AJ, et al. Incidence of Anterior Cruciate Ligament Tears and Reconstruction: A 21-Year Population-Based Study. Am J Sports Med. 2016;44(6):1502–1507.

4. Dallo I, Chahla J, Mitchell JJ, et al. Biologic approaches for the treatment of partial tears of the anterior cruciate ligament: a current concepts review. Orthop J Sports Med. 2017;5(11):232558711771274.

5. Herzog MM, Marshall SW, Lund JL, et al. Trends in Incidence of ACL Reconstruction and Concomitant Procedures Among Commercially Insured Individuals in the United States, 2002-2014. Sports Health. 2018;10(6):523–531.

6. Marshall NE, Keller RA, Dines J, Bush-Joseph C, Limprisvasti O. Current practice: postoperative and return to play trends after ACL reconstruction by fellowship-trained sports surgeons. Musculoskeletal Surg. 2019;103(1):55–61.

7. Janssen RP, Scheller SU. Intra-articular modelling of hamstring tendon grafts after anterior cruciate ligament reconstruction. Knee Surg Sports Traumatol Arthrosc. 2014;22(9):2102–2108.

8. Sato Y, Akagi R, Akatsu Y, et al. The effect of femoral bone tunnel configuration on tendon-bone healing in an anterior cruciate ligament reconstruction: An animal study. Bone Joint Res. 2018;7(5):327–335.

9. Kuroda R, Kurosaka M, Yoshiya S, Mizuno K. Localization of growth factors in the reconstructed anterior cruciate ligament: immunohistological study in dogs. Knee Surg Sports Traumatol Arthrosc. 2000;8(2):120–126.

10. Yoshikawa T, Tohya M, Katsura T, et al. Effects of local administration of vascular endothelial growth factor on mechanical characteristics of the semitendinous tendon graft after anterior cruciate ligament reconstruction in sheep. Am J Sports Med. 2006;34(12):1918–1925.

11. Kanaya A, Deim M, Adachi N, et al. Intra-articular injection of mesenchymal stromal cells in partially torn anterior cruciate ligaments in a rat model. Arthroscopy. 2007;23(6):610–617.

12. Centeno CJ, Pitts J, Al-Sayegh H, Freeman MD. Anterior cruciate ligament tears treated with percutaneous injection of autologous bone marrow nucleated cells: a case series. J Pain Res. 2015;8:437–447.

13. Matsumoto T, Kubo S, Sasaki K, et al. Acceleration of tendon-bone healing of anterior cruciate ligament graft using autologous ruptured tissue. Am J Sports Med. 2012;40(6):1296–1302.

14. Hong L, Li X, Zhang H, et al. Anterior cruciate ligament reconstruction with remnant preservation: a prospective, randomized controlled study. Am J Sports Med. 2012;40(12):2747–2755.

15. Hu J, Du J, Xu D, et al. Clinical outcomes of remnant preserving augmentation in anterior cruciate ligament reconstruction: a systematic review. Knee Surg Sports Traumatol Arthrosc. 2014;22(9):1976–1985.

16. Wu B, Zhao Z, Li S, Sun L. Preservation of remnant attachment improves graft healing in a rabbit model of anterior cruciate ligament reconstruction. Arthroscopy. 2013;29(8):1382–1371.

17. Matsumoto T, Ingham SM, Mifune Y, et al. Isolation and characterization of human anterior cruciate ligament-derived vascular stem cells. Stem Cells Dev. 2012;21(6):859–872.

18. Mifune Y, Matsumoto T, Oto S, et al. Therapeutic potential of anterior cruciate ligament-derived stem cells for anterior cruciate ligament reconstruction. Cell Transplant. 2012;21(8):1651–1655.

19. Wang CJ. Extracorporeal shockwave therapy in musculoskeletal disorders. J Orthop Surg Res. 2012;7:11.

20. Li H, Xiong Y, Zhou W, et al. Shock-wave therapy improved outcome with plantar fasciitis: a meta-analysis of randomized controlled trials. Arch Orthop Trauma Surg. 2019;139(12):1763–1770.

21. Yan C, Xiong Y, Chen L, et al. A comparative study of the efficacy of ultrasoons and extracorporeal shock wave in the treatment of tennis elbow: a meta-analysis of randomized controlled trials. J Orthop Surg Res. 2019;14(1):248.

22. Ma H, Zhang W, Shi J, Zhou D, Wang J. The efficacy and safety of extracorporeal shockwave therapy in knee osteoarthritis: A systematic review and meta-analysis. Int J Surg. 2020;75:24–34.

23. Ogden JA, Toth-Kischkat A, Schultheiss R. Principles of shock wave therapy. Clin Orthop Relat Res. 2001;387:8–17.

24. Kraivis R, Berta L. Biological effects of extracorporeal shock wave on fibroblasts. A review. Muscles Ligaments Tendons J. 2011;1(4):138–147.

25. Feichtinger X, Monforte X, Kebil C, et al. Substantial Biomechanical Improvement by Extracorporeal Shockwave Therapy After Surgical Repair of Rotator Cuff Tears. Am J Sports Med. 2019;47(9):2158–2166.

26. Langendorf EK, Klein A, Drees P, et al. Exposure to radial extracorporeal shockwaves induces muscle regeneration after muscle injury in a surgical rat model. J Orthop Res. 2019;16.

27. Hashimoto S, Ichinose T, Ohsawa T, Koibuchi N, Chikuda H. Extracorporeal Shockwave Therapy Accelerates the Healing of a Meniscal Tear in the Avascular Region in a Rat Model. Am J Sports Med. 2019;47(12):2937–2944.

28. Moretti B, Iannone F, Notarnicola A, et al. Extracorporeal shock waves down-regulate the expression of interleukin-10 and tumor necrosis factor-alpha in osteoarthritic chondrocytes. BMC Musculoskelet Disord. 2009;9:16.

29. Renz H, Rupp S. Effects of shock waves on chondrocytes and their relevance in clinical practice. Arch Orthop Trauma Surg. 2009;129(5):641–647.

30. Leone L, Vetromani M, Ranieri D, et al. Extracorporeal Shock Wave Treatment (ESWT) improves in vitro functional activities of ruptured human tendon-derived tenocytes. PLoS One. 2012;7(11):e49759.

31. Vetromani M, d’Alessandro F, Torrisi MR, et al. Extracorporeal shock wave therapy promotes cell proliferation and collagen synthesis in primary cultured human tenocytes. Knee Surg Sports Traumatol Arthrosc. 2011;19(12):2159–2168.

32. Hexter AT, Thangarajah T, Blunn G, Haddad FS. Biological augmentation of graft healing in anterior cruciate ligament reconstruction: a systematic review. Bone Joint J. 2018;100-B(3):271–284.

33. Wang CJ, Ko J-Y, Chou W-Y, et al. Shockwave therapy improves anterior cruciate ligament reconstruction. J Surg Res. 2014;188(1):110–118.

34. Wang CJ, Wang FS, Yang KD, et al. The effect of shock wave treatment at the tendon-bone interface-an histomorphological and biomechanical study in rabbits. J Orthop Res. 2005;23(3):274–282.

35. Kim KI, Park S, Im GI. Osteogenic differentiation and angiogenesis with cocultured adipose-derived stromal cells and bone marrow stromal cells. Biomaterials. 2014;35(17):4792–4804.

36. Song GY, Zhang J, Li X, Li Y, Feng H. Biomechanical and Biological Findings Between Acute Anterior Cruciate Ligament Reconstruction With and Without an Augmented Remnant Repair: A Comparative in Vivo Animal Study. Arthroscopy. 2016;32(2):307–319.

37. Lu C-C, Zhang T, Reisdorf RL, et al. Biological analysis of flexor tendon repair-failure stump tissue: A potential recycling of tissue for tendon regeneration. Bone Joint Res. 2019;8(6):232–236.

38. Zhang J, Kang N, Yu X, Ma Y, Pang X. Radial Extracorporeal Shock Wave Therapy Enhances the Proliferation and Differentiation of Neural Stem Cells by Notch, PI3K/AKT, and Wnt/beta-catenin Signaling. Sci Rep. 2017;7(1):15321.

39. Lee F-Y, Zhen Y-Y, Yuan C-M, et al. The mTOR-FAK mechanotransduction signaling axis for focal adhesion maturation and cell proliferation. Am J Transl Res. 2017;9(4):1603–1617.
EXTRACORPOREAL SHOCK WAVE PROMOTES ACTIVATION OF ANTERIOR CRUCIATE LIGAMENT REMNANT CELLS

40. Chen Y, Xu J, Huang Z, et al. An Innovative Approach for Enhancing Bone Defect Healing Using PLGA Scaffolds Seeded with Extracorporeal-shock-wave-treated Bone Marrow Mesenchymal Stem Cells (BMCs). Sci Rep 2017;7:44130.

41. Aschermann I, Noor S, Venturrelli S, et al. Extracorporeal Shock Waves Activate Migration, Proliferation and Inflammatory Pathways in Fibroblasts and Keratinocytes, and Improve Wound Healing in an Open-Label, Single-Arm Study in Patients with Therapy-Refactory Chronic Leg Ulcers. Cell Physiol Biochem 2017;41(3):890–906.

42. Rickert M, Jung M, Adiyaman M, Richter W, Simank HG. A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats. Growth Factors. 2011;29(2):115–126.

43. Spindler KP, Murray MM, Detwiler KB, et al. The biomechanical response to doses of TGF-beta 2 in the healing rabbit medial collateral ligament. J Orthop Res. 2003;21(2):245–249.

44. Wang FS, Yang KD, Chen RF, Wang CJ, Sheen-chen SM. Extracorporeal shock wave promotes growth and differentiation of bone-marrow stromal cells towards osteoprogenitors associated with induction of TGF-beta1. J Bone Joint Surg Br. 2002;84-B(3):457–461.

45. Yang G, Crawford RC, Wang JH. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. J Biomech. 2004;37(10):1543–1550.

46. Heinemeier K, Langberg H, Olsen JL, Kjaer M. Role of TGF-beta in relation to exercise-induced type I collagen synthesis in human tendinous tissue. J Appl Physiol. 2003;95(6):2390–2397.

47. Wang R, Xu B, Xu H-G. Up-Regulation of TGF-β promotes Tendon-to-Bone healing after anterior cruciate ligament reconstruction using bone marrow-derived mesenchymal stem cells through the TGF-β/SMAD signaling pathway in a new Zealand white rabbit model. Cell Physiol Biochem. 2017;41(1):213–226.

48. McCarrel T, Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. J Orthop Res. 2009;27(8):1033–1042.

49. DesRosiers EA, Yahia L, Rivard CH. Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. J Orthop Res. 1996;14(2):200–206.

50. Marui T, Niibizi C, Georgescu H, et al. Effect of growth factors on matrix synthesis by ligament fibroblasts. J Orthop Res. 1987;15(1):18–23.

51. Wang CJ, Wang FS, Yang KD, et al. Shock-wave therapy induces neovascularization at the tendon-bone junction. A study in rabbits. J Orthop Res. 2003;21(6):984–989.

52. Gollmann-Tepokoyu C, Lobenwein D, Theurl M, et al. Shock Wave Therapy Improves Cardiac Function in a Model of Chronic Ischemic Heart Failure: Evidence for a Mechanism Involving VEGF Signaling and the Extracellular Matrix. J Am Heart Assoc. 2016;7(2):e010025.

53. Hatanaka K, Ikino T, Shindo T, et al. Molecular mechanisms of the angiogenic effects of low-energy shock wave therapy: roles of mechanotransduction. Am J Physiol Cell Physiol. 2010;301(3):C376–C385.

54. Rosso F, Bonasia DE, Marmotti A, Cottino U, Rossi R. Mechanical Stimulation (Pulsed Electromagnetic Fields “PEMF” and Extracorporeal Shock Wave Therapy “ESWT”) and Tendon Regeneration: A Possible Alternative. Front Aging Neurosci. 2015;7:211.

55. Suhr F, Delhaes Y, Bugnartz G, et al. Cell biological effects of mechanical stimulations generated by focused extracorporeal shock wave applications on cultured human bone marrow stromal cells. Stem Cell Res. 2013;11(2):951–964.

56. Johannes EJ, Kaulesar Sukul DM, Matura E. High-energy shock waves for the treatment of nonunions: an experiment on dogs. J Surg Res. 1994;57(2):246–252.

57. Haupt G, Chvapil M. Effect of shock waves on the healing of partial-thickness wounds in piglets. J Surg Res. 1990;49(1):45–48.

58. Chen YJ, Wang CJ, Yang KD, et al. Extracorporeal shock waves promote healing of collagenase-induced Achilles tendinitis and increase TGF-beta1 and IGF-I expression. J Orthop Res. 2004;22(4):854–861.

Author information:

© C-C. Lu, MD, Orthopaedic Surgeon, Department of Orthopedics, Kaohsiung Municipal Siagang Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan; Department of Orthopedics, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; Regenerative Medicine and Cell Therapy Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan.

H.-H. Chou, MD, Orthopaedic Surgeon

P.-C. Shen, MD, Orthopaedic Surgeon

Department of Orthopedics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan.

P.-H. Chou, PhD, Professor, Orthopaedic Surgeon

Department of Orthopedics, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; Department of Orthopedics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan.

M.-L. Ho, PhD, Professor, Regenerative Medicine and Cell Therapy Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan; Department of Physiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

Author contributions:

C-C. Lu: Performed the experiments, Analyzed the data, Wrote the manuscript.

H.-H. Chou: Analyzed the data.

P.-C. Shen: Performed the experiments.

P.-H. Chou: Designed the research studies, Edited the manuscript.

M.-L. Ho: Edited the manuscript.

Y.-C. Tien: Designed the research studies, Analyzed the data, Edited the manuscript.

Funding statement:

This study was supported by grants from Kaohsiung Medical University Hospital (No. KMUHO15-SM33), the Ministry of Science and Technology, Taiwan (MOST 109-2314-B-037-016-MY2 ), and Regenerative Medicine and Cell Therapy Research Center (KMU-TC108A02-667). No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

ICMJE COI statement:

The investigators declare no conflicts of interest.

Acknowledgements

The authors acknowledged the grants from Kaohsiung Medical University Hospital (No. KMUH105-5M33), the Ministry of Science and Technology, Taiwan (MOST 109-2314-B-037-016-MY2), and Regenerative Medicine and Cell Therapy Research Center (KMU-TC108A02-667). We also acknowledged Miss Yu-Han Lin for statistical analysis.

Ethical review statement:

This study was approved by the Kaohsiung Medical University Hospital Institutional Review Board (IRB) (KMUH108-F102-20160112) and Institutional Animal Care and Use Committee (IACUC) (KMUH105263).

© 2020 Author(s) et al. This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (CC BY-NC-ND 4.0) licence, which permits the copying and redistribution of the work only, and provided the original author and source are credited. See https://creativecommons.org/licenses/by-nc-nd/4.0/.