**Original Article**

# The effects of treatment using polydeoxyribonucleotide through extracorporeal shock wave therapy: synergic regeneration effects on atrophied calf muscles in immobilized rabbits

**Kang Lip Kim[1], Gi Young Park[1], Yong Suk Moon[2], Dong Rak Kwon[1]**

[1]Department of Rehabilitation Medicine, Catholic University of Daegu School of Medicine, Daegu, South Korea; [2]Department of Anatomy, Catholic University of Daegu School of Medicine, Daegu, South Korea

*Contributions:* (I) Conception and design: DR Kwon; (II) Administrative support: DR Kwon; (III) Provision of study materials or patients: DR Kwon; (IV) Collection and assembly of data: DR Kwon, YS Moon, KL Kim; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*Correspondence to:* Dong Rak Kwon, MD, PhD. Department of Rehabilitation Medicine, Catholic University of Daegu School of Medicine, 33 Duryugongwon-ro 17-gil, Nam-Gu, Daegu 42472, South Korea. Email: coolkwon@cu.ac.kr or dongrakkwon@hotmail.com.

**Background:** This study aimed to examine the synergic effects of polydeoxyribonucleotide (PDRN) through extracorporeal shock wave therapy (ESWT) on atrophied calf muscles in cast-immobilized rabbit models.

**Methods:** Twenty male New Zealand rabbits (aged 12 weeks) were allocated into four groups. Four types of procedures [0.7 mL normal saline to Group 1 (G1-NS); 0.7 mL PDRN to Group 2 (G2-PDRN); ESWT to Group 3 (G3-ESWT); and 0.7 mL PDRN with ESWT to Group 4 (G4-PDRN + ESWT)] were injected to the atrophied calf muscles of the rabbits after two weeks of cast immobilization. Radial ESWT (0.1 mJ/mm$^2$, 3 Hz, 1,500 shocks) was performed twice weekly. The circumference of the calves, compound muscle action potential (CMAP) of the tibial nerves, and thickness of the gastrocnemius (GCM) muscle were evaluated after two weeks of treatment. Type I and II GCM muscle fibers were immunohistochemically stained using monoclonal anti-myosin, anti-VEGF (vascular endothelial growth factor), and anti-PECAM-1 (platelet endothelial cell adhesion molecule-1) antibodies, and the cross-sectional area (CSA), VEGF ratio, and PECAM ratio were measured after 2 weeks of treatment. Statistical differences among the four groups were determined using analysis of variance (ANOVA).

**Results:** The G4-PDRN + ESWT group had a significantly greater circumference of calf muscles, thickness of the GCM muscle, CMAP of the tibial nerve, and CSA of the GCM muscle fibers (type I, II, and total) (hereinafter termed "the four categories") than those in the remaining three groups (P<0.05). Rabbits in the G3-ESWT group had significantly higher results in the four categories than in G1-NS and G2-PDRN groups (P<0.05). G2-PDRN rabbits had significantly higher results in the four categories than those in G1-NS (P<0.05). The VEGF and PECAM-1 ratio of the medial GCM muscle fibers in G4-PDRN + ESWT were significantly higher than those in the remaining three groups (P<0.05).

**Conclusions:** ESWT combined with PDRN injection was more effective in muscle regeneration than ESWT, PDRN injection alone, or normal saline injection on atrophied calf muscles in rabbit models.

**Keywords:** Atrophy; polydeoxyribonucleotides (PDRNs); extracorporeal shock waves therapy; muscle; rabbit

Page 2 of 14

Kim et al. PDRN and shock wave on muscle atrophy.

## Introduction

Atrophy refers to a condition wherein the volume of a normal body part is partially or completely reduced. The etiology of atrophy includes malnutrition, inability to control the nerves in the target organs, lack of exercise, and immobilization. In addition, a prior study reported the regression of the capillary network, which is essential for bringing nutrients and immune cells to heal and regenerate atrophied muscles (1). Many types of injuries, such as ligament or tendon injuries, fractures, or recovery after orthopedic surgery, require a certain period of physical inactivity in the process of patient recovery (2). Immobilization causes various negative effects on health, such as decreased myofibrillar proteins amount, reduced metabolic enzyme function, vascular and neural alterations, and abnormal ectopic fat accumulation in muscles (3). Therefore, it is necessary to establish an appropriate intervention to diminish atrophy during immobilization.

Polydeoxyribonucleotide (PDRN) is a mixture of deoxyribonucleotide polymer chains. PDRN contributes to anti-inflammatory activity by reducing the expression of inflammatory cytokines such as tumor necrosis factor-alpha and increasing the expression of anti-inflammatory cytokines, including interleukin-10 (4,5). Prior studies have reported that PDRN promoted the generation and release of vascular endothelial growth factor (VEGF) by invigorating the effect of adenosine on $A_{2A}$ receptors (6,7). Through this process, PDRN can stimulate new blood vessel formation and collagen synthesis.

Extracorporeal shock wave therapy (ESWT) involves using acoustic waves generated from the body and that are focused upon treating a particular body part. A previous study reported that ESWT decreased the pain associated with peripheral nerve injury and induced arterial remodeling (8). A recent study also investigated the pain-decreasing effect of ESWT and suggested that it stimulates the regeneration of axons (9). ESWT is a therapeutic tool for various musculoskeletal problems, including calcific tendinopathy of the rotator cuff, lateral epicondylitis, spasticity, and plantar fasciitis (10-13). A recent systematic review showed the effectiveness of ESWT was comparable to botulinum toxin injections for the treatment of post-stroke spasticity (13). However, few studies have proven that ESWT can contribute to muscle regeneration from muscle injuries or muscle atrophy. Lee *et al.* reported that ESWT could be a treatment option to improve the function of the sciatic nerve and prevent denervation atrophy in a rat model. Compared to the control group, the ESWT group showed a significant increase in the sciatic functional index score after treatment (14). Another rat model-based study suggested that ESWT could promote the repair and regeneration of skeletal muscle tissues after skeletal muscle injury (15). The authors postulated that ESWT contributed to muscle regeneration through mechanotransduction and increasing the gene expression of multiple growth factors such as insulin-like growth factor, fibroblast growth factor, and VEGF.

The objective of this study was to assess the synergistic regenerative effects of PDRN injection combined with ESWT on atrophied calf muscle in cast-immobilized rabbits. We believe that this study is the first to investigate such synergistic regenerative effects. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-854/rc).

## Methods

### Animal model and grouping

This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Daegu School of Medicine (IRB No. DCIAFCR-200921-10-Y), which is the author's affiliation, in compliance with IACUC guidelines for the care and use of animals. We housed male New Zealand White rabbits (n=20, 12 weeks old), weighing 3.3 kg (2.8–3.6 kg) on average in separate steel cages at constant temperature and humidity (23±2 ℃ and 45%±10%, respectively). All rabbits were allowed free access to tap water and were fed a commercial rabbit diet.

We then randomly allocated rabbits into four groups (n=5 per group). All rabbits were assigned a number from one to twenty, and then allocated into four groups using the program for random grouping (Research Randomizer). After two weeks of immobilization using a cast (IC), we removed the IC. We then removed the hair in the lower limbs of the rabbits using a commercial hair remover. We performed four types of procedures on their atrophied calf muscles (*Figure 1*): Group 1—normal saline, 0.7 mL injection (G1-NS); Group 2—PDRN, 0.7 mL injection (G2-PDRN); Group 3—ESWT (G3-ESWT); and Group 4—0.7 mL PDRN injection with ESWT (G4-PDRN + ESWT).
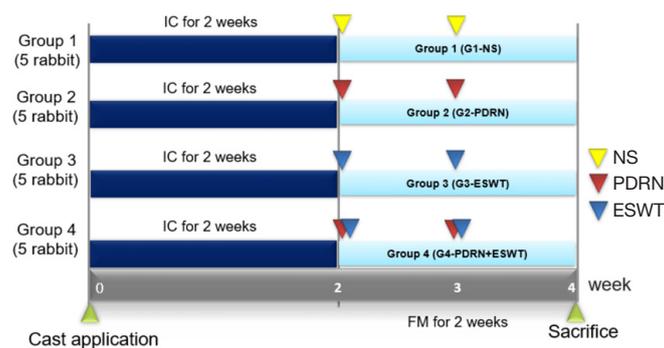
**Figure 1** Timeline of the study. Twenty rabbits were randomly allocated to 4 groups. G1-NS, IC for 2 weeks and 0.7 mL normal saline injection for 2 weeks after CR; G2-PDRN, IC for 2 weeks and 0.7 mL PDRN injection for 2 weeks after CR; G3-ESWT, IC for 2 weeks and ESWT after CR; G4-PDRN + ESWT, IC for 2 weeks and 0.7 mL PDRN injection and ESWT for 2 weeks after CR. IC, immobilized by cast; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; FM, free movement; CR, cast removal.

### Immobilization by cast

We immobilized all the rabbits' right lower limbs using IC for two weeks. Based on a cast immobilization procedure described in previous report (16), we extended the right knees and ankles using a splint. The splint was made of an adhesive elastic bandage and PVC, a non-adhesive bandage (Tensoplast®; Smith & Nephew Medical, London, UK).

### Injection procedures

All injection procedures were performed under anesthesia with Zoletil® 50 (15 mg/kg, Virbac Korea, Seoul, South Korea) and xylazine (5 mg/kg, Rompun®; Bayer Co., Seoul, South Korea) via intramuscular injection. We administered normal saline or PDRN injections (0.7 mL respectively) at two points, the lateral and the medial side of the gastrocnemius (GCM) muscle, under ultrasound guidance with a 5–13-MHz multifrequency linear transducer (Antares; Siemens Healthcare, Erlangen, Germany) (*Figure 2A,2B*). We used commercially obtained PDRN (Rejuvenex Inj., PDRN sodium, 5.625 mg/3 mL, Pharma Research Product, South Korea). We injected 0.35 mL of each solution into the lateral and medial sides on the same horizontal line based on a middle reference point (a total of 0.7 mL in two points). The middle reference point was determined as the midpoint between these two points: the proximal one-third point of a longitudinal line drawn from the midpoint between the malleoli of both ankles to the midpoint between the femoral epicondyles, and the medial and lateral endpoints of a transverse line drawn

perpendicularly to the point of the longitudinal line. The injection was repeated at the same sites one week after the initial intervention.

### ESWT application

We used a radial-type machine for ESWT (BTL-5000; BTL, Columbia, SC, USA) and applied pressure pulses on the lateral and medial sides of the GCM muscle. For Group 4 (G4-PDRN + ESWT), we performed ESWT immediately after PDRN injection (*Figure 2C,2D*). We used a transmission gel as the contact medium for applying ESWT. We applied 750 shocks to each injection site, for a total of 1,500 shocks (two sites; energy density =0.1 mJ/mm², frequency =3 pulses/s). We repeated ESWT at the same sites one week after the initial intervention.

### Clinical parameters

All the parameters of the study were measured by a physiatrist who was unaware of the group arrangement, and the physiatrist had many years of professional experiences in musculoskeletal ultrasound (18 years) and electrophysiological studies (23 years). Prior to euthanasia, we performed a motor nerve conduction study to evaluate the amplitude of the compound muscle action potential (CMAP) on the tibial nerve. Briefly, we placed the active electrode at the midpoint of the GCM muscle by keeping the reference electrode on the subcutaneous tissue at the ankle. We then conducted electrical stimulation of the tibial nerve in the popliteal fossa. We recorded the highest

Page 4 of 14

Kim et al. PDRN and shock wave on muscle atrophy.
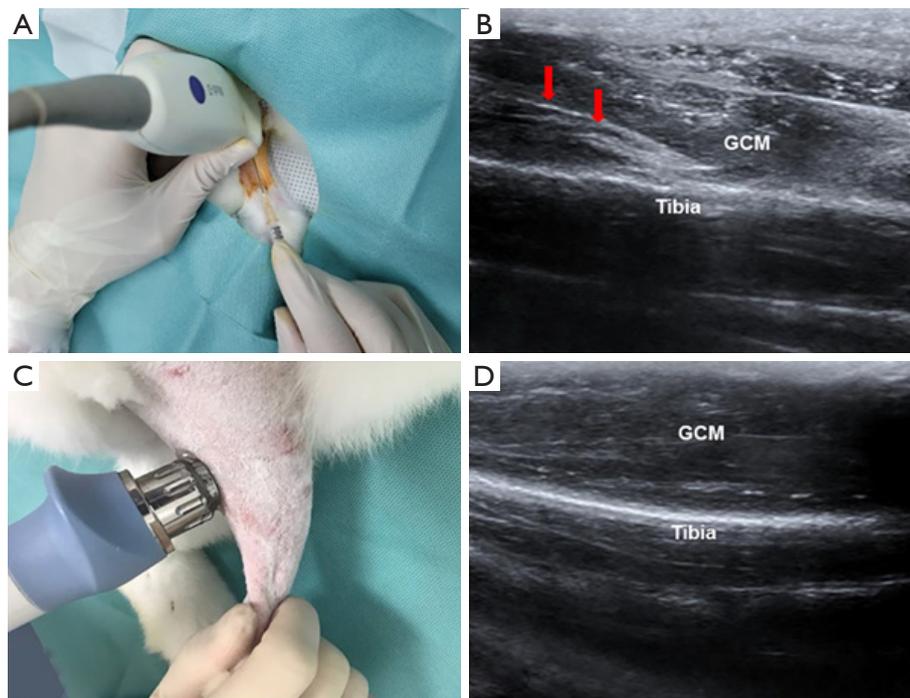


**Figure 2** Longitudinal ultrasound image showing the injection of 0.7 mL PDRN (A) under ultrasound guidance (needle, red arrows) in the right gastrocnemius muscles of a rabbit (B). ESWT (C) was then performed under ultrasonic guidance to the region of interest, focusing on the PDRN injection area (D). GCM, gastrocnemius; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy.

CMAP after 7–10 supramaximal stimuli.

We measured the largest circumference of the calf areas using a tape measure. During the measurement, we flexed the knee joints of the rabbits at a 90° angle with the ankle relaxed. We assessed the thickness of the lateral and medial GCM from the superficial to the deep fascia of the GCM using real-time B-mode ultrasound. We acquired longitudinal ultrasound images of the GCM at fixed points similar to the injection sites, at the lateral and medial surfaces of the muscle.

Finally, we evaluated the atrophic changes in CMAP amplitude, the thickness of the GCM, and calf circumference using the following equation: [(left side – right side)/left side × 100]. We presented the results as a percentage of atrophic changes on the right side compared to the left side.

### Tissue preparation

All the histological parameters of the study were measured by an anatomist who was unaware of the group arrangement. All rabbits were euthanized at two weeks after

IC removal under general anesthesia after all intramuscular injections. Muscle samples were collected from the right GCM for the microscopic assessment. We segmented the lateral and medial GCM muscular fibers and fixed them with neutral-buffered formalin for a day. We embedded the specimens in paraffin (Paraplast; Oxford, St. Louis, MO, USA). Then, the specimens were sliced into 5-mm thick transverse sections.

### Immunohistochemical analysis

We immunohistochemically stained muscle sections for type I and type II fibers with monoclonal anti-myosin antibodies (Sigma-Aldrich, St. Louis, MO, USA). A Monoclonal anti-myosin antibody (skeletal, slow) was used for type I fibers, and a monoclonal anti-myosin antibody (skeletal, fast) was used for type II fibers. The sections were then immunostained with angiogenic markers using a polyclonal anti-VEGF antibody (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-PECAM-1 polyclonal antibody (M-20; Santa Cruz Biotechnology). The paraffin-embedded sections were cleared and dehydrated. Then, we

washed the sections using phosphate-buffered saline (PBS). We inhibited endogenous peroxidases for 30 min by pre-incubation in 0.3% $H_2O_2$ in PBS. Non-specific protein binding was blocked upon treatment with PBS containing 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 30 min. We incubated the sections with the corresponding primary antibodies (1:200–1:500) at room temperature for 2 h. Then, we washed the sections three times using PBS. Afterward, the sections were incubated with the corresponding secondary antibody (1:100). Then, biotinylated anti-mouse IgG (Vector Laboratories) was added to the muscle sections for 1 h at room temperature. The sections were subsequently washed three times with PBS. We then incubated the sections with an avidin-biotin-peroxidase complex (ABC, Vector Laboratories) for 1 h and washed them three times with PBS, followed by a peroxidase reaction using 0.05 M Tris-HCl (pH 7.6) containing 0.01% $H_2O_2$ and 0.05% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). We counterstained the sections using hematoxylin and mounted them on slides. Finally, we inspected the slides by an Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany) with an Axio-Cam MRc5 attachment (Carl Zeiss).

We conducted all histologic analyses by being blinded to the entire study process, including the group allocation. Muscle sections were examined using an Axiophot Photomicroscope (Carl Zeiss). We captured five randomly selected fields from each group. We extracted the entire muscle cross-section from the digital images of the anti-myosin immunostained muscle sections (×100 magnification). We then measured the cross-sectional area (CSA) of the anti-myosin positive type I and II muscle fibers using an image morphometry program (AxioVision SE64; Carl Zeiss) and then measured the mean value.

To examine VEGF and PECAM-1 immunostaining, we took photos of 20 randomly selected fields from each group and analyzed them using AxioVision SE64 software (Carl Zeiss). We counted the number of VEGF- and PECAM-1-positive cells or nuclei and the total number of muscle fibers within each image. We applied the ratio of the number of VEGF- and PECAM-1-positive cells or nuclei per 1,000 muscle fibers to report the VEGF and PECAM-1 ratios, respectively.

### Statistical analysis

All statistical analyses were performed using SPSS v22.0 for Windows (IBM Corp., USA). P values <0.05 were considered significant. For calculating sample size, we conducted pilot study. The primary end point is total muscle fiber CSA of medial GCM. In pilot study, we used one rabbit in each group, therefore five randomly selected field was evaluated in each group. The effect size was 0.49, and for such effect size, to achieve a power of at least 95% using the analysis of variance (ANOVA) with a significance level of 0.05, at least 76 fields were needed. Five fields can be obtained from one rabbit. Therefore, 16 rabbits were needed. Considering drop rate as 20%, we determined sample size as 20. We used standard descriptive statistical calculations for means and standard errors and ANOVA to identify statistical differences between the four groups. When significant differences among groups appeared in the ANOVA analysis, we performed Tukey's test. We used 95% confidence intervals to indicate the precision of the mean values obtained.

## Results

We found significant differences in the imaging, electrophysiological, and clinical parameters between the G1-NS and the other groups (P<0.05, *Table 1*). The mean atrophic changes (%) in the right medial and lateral GCM muscle thickness (*Figure 3*), right calf circumference, and CMAP amplitude of the right tibial nerve in the G4-PDRN + ESWT group were significantly lower than those in the other three groups (P<0.05, *Table 1*, *Figure 4*). The mean atrophic changes in calf muscle circumference, GCM muscle thickness, and CMAP amplitude of the tibial nerve in the G3-ESWT group were significantly lower than those in the G1-NS and G2-PDRN groups (P<0.05, *Table 1*, *Figure 4*). The mean atrophic changes in calf muscle circumference, GCM muscle thickness, and CMAP amplitude of the tibial nerve in the G2-PDRN group were significantly lower than those in the G1-NS group (P<0.05, *Table 1*, *Figure 4*).

Immunohistochemical analysis between the G1-NS and the other groups (P<0.05, *Table 2*, *Figure 5*) showed significant differences. The mean CSA of the type I medial and lateral GCM muscle fibers in the G4-PDRN + ESWT group (1,172.14±23.7 and 1,136.6±38.0 µm², respectively) were significantly larger than those in the remaining three groups (P<0.05, *Table 2*, *Figures 6,7*). Better outcomes in the mean CSA of type II muscle fibers were also observed in the G4-PDRN + ESWT group (1,597.7±23.8 and 1,513.0±17.5 µm², respectively) than in the other three groups (P<0.05, *Table 2*, *Figures 6,7*). The mean CSA (µm²) of the medial and lateral

**Table 1** Comparison of regenerative effect of clinical parameters among four groups

| Groups | Atrophic changes (%) | | | |
|---|---|---|---|---|
| | Circumference of Rt. Calf (cm) | CMAP on Rt. Tibial nerve (mV) | Rt. GCM muscle thickness (mm) | |
| | | | Medial | Lateral |
| G1-NS | 24.1±1.6 | 25.7±2.0 | 23.6±1.3 | 24.1±0.7 |
| G2-PDRN | 19.5±1.7* | 20.5±1.1* | 19.8±0.8* | 20.3±0.9* |
| G3-ESWT | 17.0±0.7[†§] | 17.8±1.1[†§] | 17.4±1.0[†§] | 18.4±0.9[†§] |
| G4-PDRN + ESWT | 14.2±0.7[‡∥¶] | 15.1±1.3[‡∥¶] | 15.0±0.9[‡∥¶] | 15.8±1.3[‡∥¶] |

Values are presented as mean ± standard deviation. G1-NS, IC for 2 weeks and 0.2 mL normal saline injection for 2 weeks after CR; G2-PDRN, IC for 2 weeks and 0.2 mL PDRN injection for 2 weeks after CR; G3-ESWT, IC for 2 weeks and ESWT after CR; G4-PDRN + ESWT, IC for 2 weeks and 0.2 mL PDRN injection and ESWT for 2 weeks after CR. *, $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; [†], $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; [‡], $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; [§], $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 2 and 3; [∥], $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; [¶], $P<0.05$ one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. CMAP, compound muscle action potential; GCM, gastrocnemius; IC, immobilized by cast; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; CR, cast removal; ANOVA, analysis of variance.
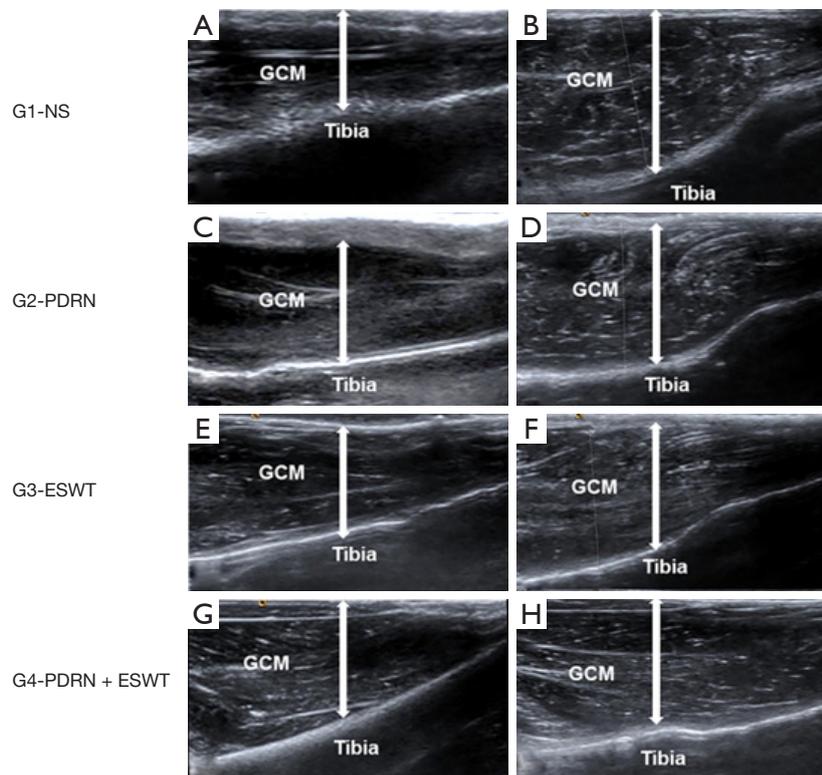


**Figure 3** GCM muscle thickness was measured via ultrasound. Thickness was measured as the distance from the superficial aponeurosis to the deep aponeurosis of the GCM muscle (up-down arrows). Representative longitudinal sonograms of the right (A,C,E,G) and left (B,D,F,H) GCM muscle. The atrophic change of the right GCM muscle in the rabbits in the G4-PDRN + ESWT group (G,H) less pronounced than that in the other three groups (A-F). NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; GCM, gastrocnemius.
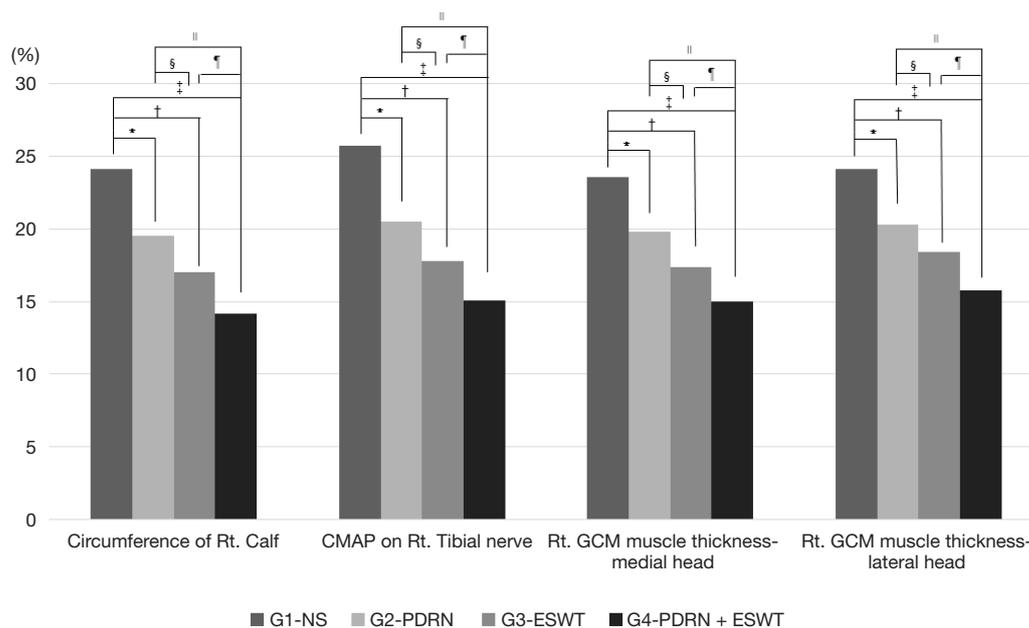
**Figure 4** Comparison of regenerative effect of clinical parameters among four groups. *, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; §, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 3; ‖, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. CMAP, compound muscle action potential; GCM, gastrocnemius; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; ANOVA, analysis of variance.

**Table 2** Comparison of immunohistochemical findings in gastrocnemius muscle fiber among four groups

| Immunohistochemical findings | G1-NS | G2-PDRN | G3-ESWT | G4-PDRN + ESWT |
|---|---|---|---|---|
| Medial GCM | | | | |
| Type I fiber CSA (μm$^2$) | 287.6±5.5 | 633.6±9.6* | 938.0±16.0$^{†§}$ | 1,172.14±23.7$^{‡‖¶}$ |
| Type II fiber CSA (μm$^2$) | 443.1±4.5 | 955.1±13.9* | 1,257.3±16.7$^{†§}$ | 1,597.7±23.8$^{‡‖¶}$ |
| Total muscle fiber CSA (μm$^2$) | 399.0±20.5 | 888.3±12.1* | 1,197.2±14.5$^{†§}$ | 1,509.3±20.5$^{‡‖¶}$ |
| VEGF ratio | 0.17±0.07 | 0.30±0.12* | 0.29±0.11$^†$ | 0.40±0.11$^{‖¶}$ |
| PECAM-1 ratio | 0.08±0.05 | 0.30±0.08* | 0.31±0.10$^†$ | 0.39±0.06$^{‖¶}$ |
| Lateral GCM | | | | |
| Type I fiber CSA (μm$^2$) | 286.2±12.3 | 538.71±14.5* | 843.22±25.3$^{†§}$ | 1,136.6±38.0$^{‡‖¶}$ |
| Type II fiber CSA (μm$^2$) | 420.1±4.9 | 1,063.0±13.0* | 1,207.4±14.4$^{†§}$ | 1,513.0±17.5$^{‡‖¶}$ |
| Total muscle fiber CSA (μm$^2$) | 400.73±4.8 | 973.9±13.2* | 1,172.3±13.7$^{†§}$ | 1,466.0±16.6$^{‡‖¶}$ |
| VEGF ratio | 0.15±0.05 | 0.31±0.09* | 0.32±0.06$^†$ | 0.42±0.08$^{‡‖¶}$ |
| PECAM-1 ratio | 0.08±0.06 | 0.30±0.05* | 0.31±0.09$^†$ | 0.39±0.08$^{‡‖¶}$ |

Values are presented as mean ± standard deviation. G1-NS, IC for 2 weeks and 0.7 mL normal saline injection for 2 weeks after CR; G2-PDRN, IC for 2 weeks and 0.7 mL PDRN injection for 2 weeks after CR; G3-ESWT, IC for 2 weeks and ESWT after CR; G4-PDRN + ESWT, IC for 2weeks and 0.7 mL PDRN injection and ESWT for 2 weeks after CR. *, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; §, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 3; ‖, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; GCM, gastrocnemius; CSA, cross-sectional area; VEGF, vascular endothelial growth factor; PECAM-1, platelet endothelial cell adhesion molecule-1; IC, immobilized by cast; CR, cast removal; ANOVA, analysis of variance.

**Page 8 of 14**

**Kim et al. PDRN and shock wave on muscle atrophy.**
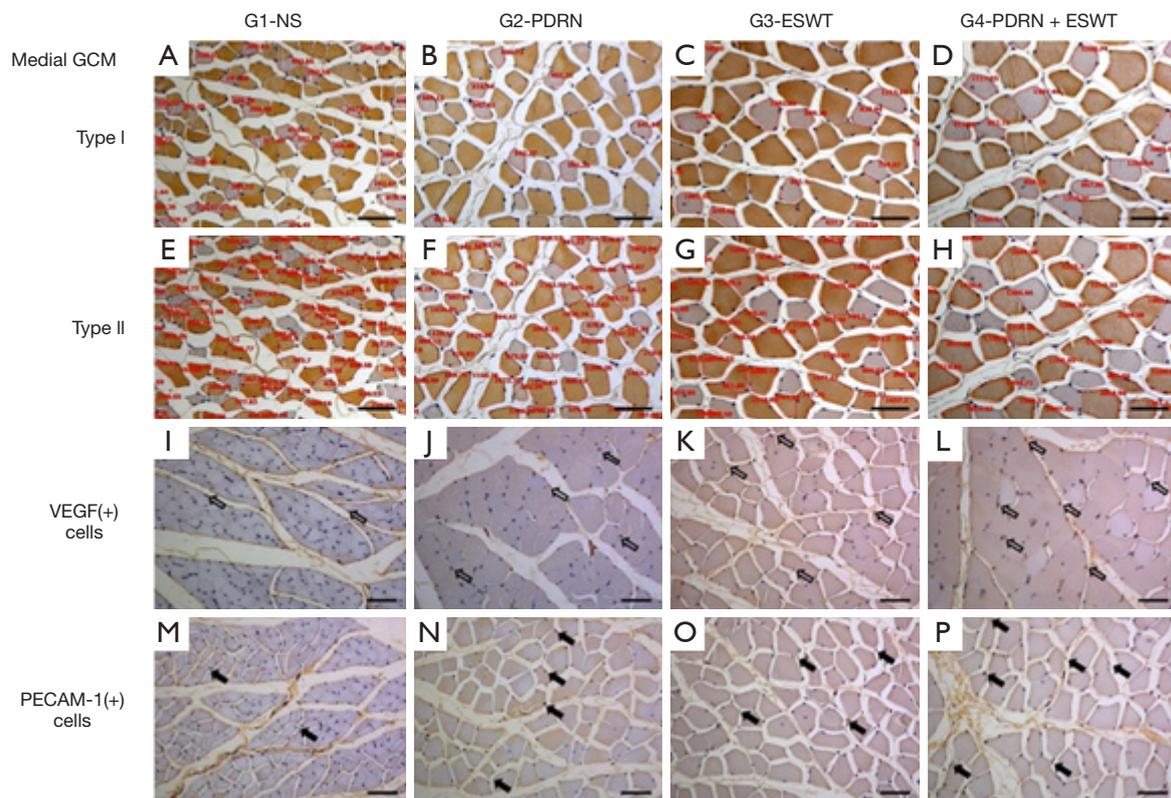
**Figure 5** Immunohistochemical findings of medial GCM muscle fibers among the four groups. Immunohistochemical findings of the immobilized medial GCM muscles stained with monoclonal anti-myosin type II (A-H), anti-VEGF (I-L), and anti-PECAM-1 (M-P) antibodies. The cross-sectional areas (red circles) of medial head of gastrocnemius type 1 (A-D) and type 2 (E-H) muscle fiber were measured using an image morphometry program. Atrophied muscle fibers were seen in G1-NS (A,E). The cross-sectional areas of muscle fibers were increased in G2-PDRN (B,F), G3-ESWT (C,G), and G4-PDRN + ESWT (D,H) as compared with G1-NS. VEGF and PECAM-1 positive cells or nuclei (arrows) and the total number of muscle fibers within each image were counted. The VEGF and PECAM-1 ratios of the medial GCM muscle fibers in G4-NS were significantly higher than those in other three groups. Scale bar is 50 μm. G1-NS, IC for 2 weeks and 0.7 mL normal saline injection for 2 weeks after CR; G2-PDRN, IC for 2 weeks and 0.7 mL PDRN injection for 2 weeks after CR; G3-ESWT, IC for 2 weeks and ESWT after CR; G4-PDRN + ESWT, IC for 2 weeks and 0.7 mL PDRN injection and ESWT for 2 weeks after CR. NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; GCM, gastrocnemius; VEGF, vascular endothelial growth factor; and PECAM-1, platelet endothelial cell adhesion molecule-1; IC, immobilization by cast.

GCM muscle fibers (type I, II, and total) in the G3-ESWT group were significantly greater than those in the G1-NS and G2-PDRN groups (P<0.05, *Table 2*, *Figures 6*,7). The mean CSA (μm$^2$) of medial and lateral GCM muscle fibers (type I, II, and total) in the G2-PDRN group were significantly greater than those in the G1-NS group (P<0.05, *Table 2*, *Figures 6*,7).

The VEGF and PECAM-1 ratios of the medial and lateral GCM muscle fibers in the G4-PDRN + ESWT group were significantly higher than those in the remaining three groups (P<0.05, *Table 2*, *Figures 8*,9). The VEGF ratios of the medial and lateral GCM muscle fibers in the

G3-ESWT (0.29±0.11 and 0.32±0.06, respectively) and G2-PDRN groups (0.30±0.12 and 0.31±0.09, respectively) were significantly higher than those in the G1-NS group (0.17±0.07 and 0.15±0.05, respectively) (P<0.05, *Table 2*, *Figures 8*,9). The PECAM-1 ratios of the medial and lateral GCM muscle fibers in the G3-ESWT (0.31±0.10 and 0.31±0.09, respectively) and the G2-PDRN groups (0.30±0.08 and 0.30±0.05, respectively) were significantly higher than those in the G1-NS group (0.08±0.05 and 0.08±0.06, respectively) (P<0.05, *Table 2*, *Figures 8*,9). The VEGF and PECAM-1 ratios between the G3-ESWT and G2-PDRN groups (*Table 2*, *Figures 8*,9) were not
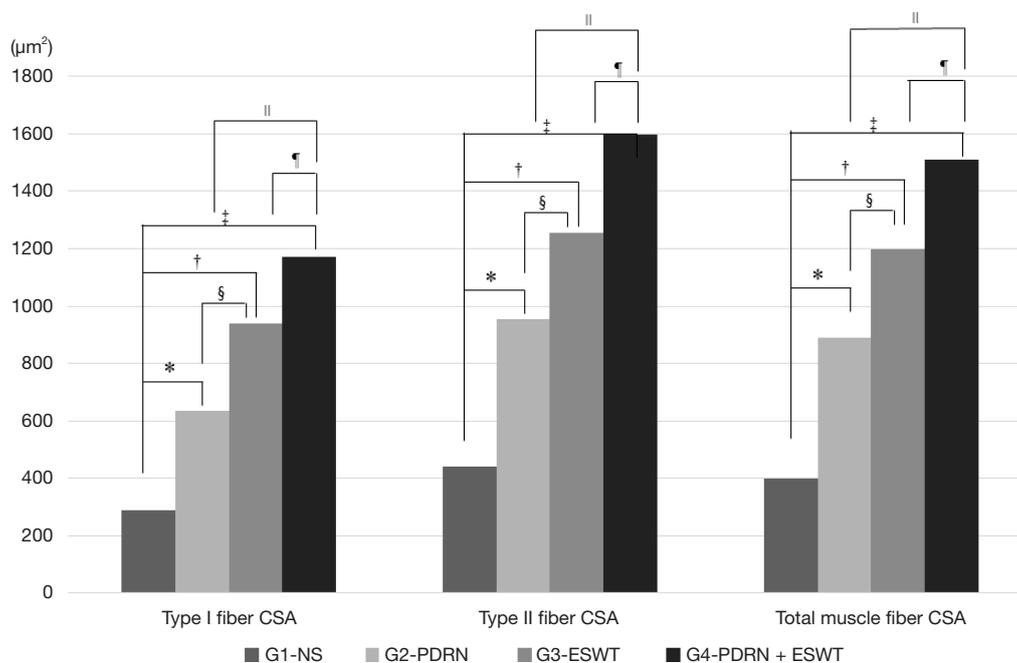
**Figure 6** Comparison of mean CSA of medial GCM muscle fibers among four groups. *, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; §, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 3; ‖, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. CSA, cross sectional area; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; GCM, gastrocnemius; ANOVA, analysis of variance.

significantly different.

## Discussion

In this study, we investigated the synergistic effects of PDRN injection combined with ESWT on the regeneration of atrophied calf muscles in cast-immobilized rabbits. Our results indicated that PDRN injection into the GCM muscle significantly reduced the atrophic change of the GCM muscle in cast-immobilized rabbits compared to those administered with normal saline. Furthermore, when PDRN injection was combined with ESWT, the atrophic change of the GCM muscle was significantly lower than PDRN injection or ESWT alone.

Clinically, ESWT is a therapeutic tool for various musculoskeletal problems, including rotator cuff calcific tendinopathy, spasticity, medial and lateral epicondylitis, and plantar fasciitis (10,11,17,18). In this study, we applied radial ESWT to the atrophied muscles of rabbits. A feature of the radial type of ESWT is its less infiltration from the body surface and less centralization of the energy to

the treatment object than the focused type (19). A recent systematic review reported the benefit of the radial type compared to the focused type of ESWT, including a larger therapeutic area, no need for accurate targeting, and no requirement for additional local anesthesia (20). In addition to these advantages, we applied the radial type of ESWT rather than the focused type because the entire GCM muscle was atrophied in the animal model we established.

We found no previous study that directly applied ESWT to treat atrophied muscle. However, one previous study applied ESWT to treat myofascial pain syndrome (MPS) (21). In that study, they conducted 1500 pulses of ESWT once a week for two weeks and applied the same procedures in the current study. That study showed that ESWT effectively improved pain and subjective disability in patients with MPS (21).

Our study showed that ESWT could contribute to the recovery from atrophy caused by immobilization. A shock wave is a sound wave that propagates a high positive pressure amplitude compared with an ultrasonic wave with limited bandwidth. When a sound wave is created by high pressure,

Page 10 of 14

Kim et al. PDRN and shock wave on muscle atrophy.
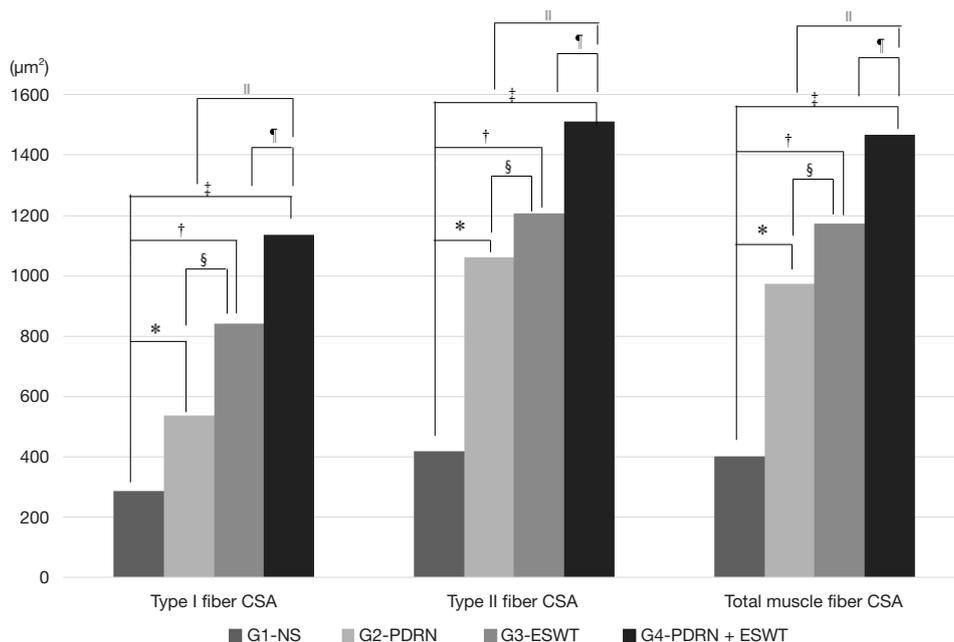


**Figure 7** Comparison of mean CSA of lateral GCM muscle fibers among four groups. *, P<0.05 one-way ANOVA, Tukey's post hoc test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; §, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 3; ||, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. CSA, cross sectional area; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; GCM, gastrocnemius; ANOVA, analysis of variance.
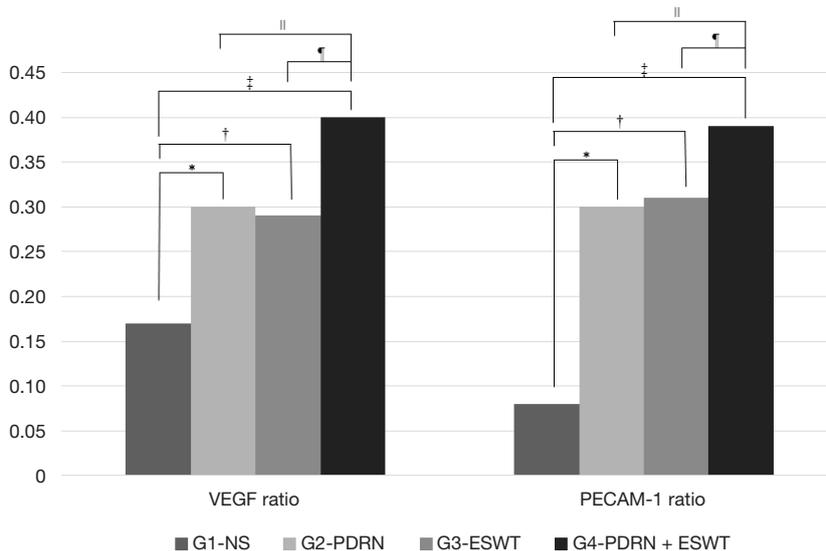


**Figure 8** Comparison of VEGF and PECAM-1 ratios of the medial GCM muscle fibers among four groups. *, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; ||, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. VEGF, vascular endothelial growth factor; PECAM-1, platelet endothelial cell adhesion molecule-1; GCM, gastrocnemius; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; ANOVA, analysis of variance.
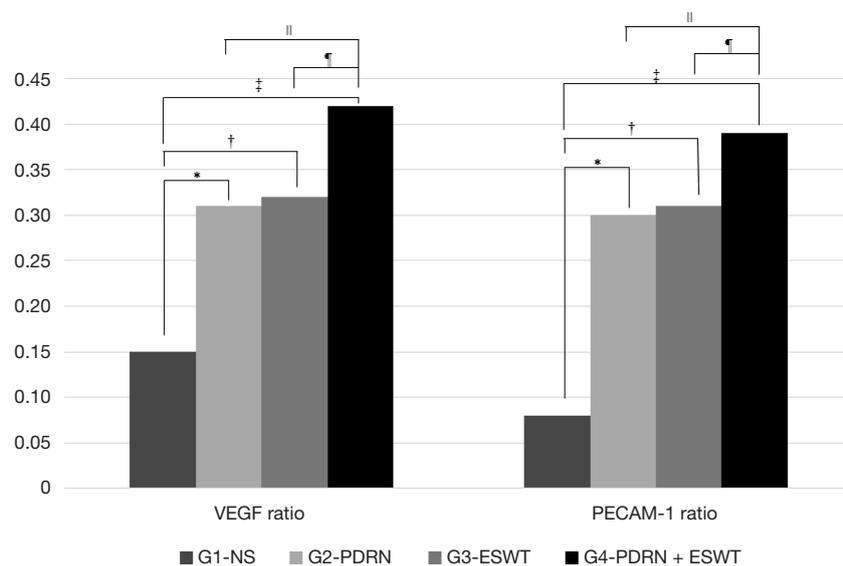
**Figure 9** Comparison of VEGF and PECAM-1 ratios of the lateral GCM muscle fibers among four groups. *, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 2; †, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 3; ‡, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 1 and 4; ‖, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 2 and 4; ¶, P<0.05 one-way ANOVA, Tukey's *post hoc* test among group 3 and 4. VEGF, vascular endothelial growth factor; PECAM-1, platelet endothelial cell adhesion molecule-1; and GCM, gastrocnemius; NS, normal saline; PDRN, polydeoxyribonucleotide; ESWT, extracorporeal shock wave therapy; ANOVA, analysis of variance.

its velocity increases and generates a high amount of energy by changing its waveform. Although the exact mechanism of ESWT is still controversial, several previous studies suggested that it could stimulate neovascularization (22) and promote the proliferation of tenocytes (23), which could contribute to a decrease in pain and the induction of functional recovery. Zhang *et al.* suggested that ESWT stimulate lubricin production in tendons and septa, and it may decrease wear and tear in tissues treated with ESWT (24). Additionally, an *in vitro* study showed that gene expression of muscle specific genes such as paired box protein 7 (Pax7), neural cell adhesion molecule (NCAM), and myogenic factor 5 (Myf5) increased significantly in the muscle cells subjected to radial ESWT compared to non-treated muscle cells (25). This study showed that the circumference of calf muscles, the thickness of the GCM muscle, the CMAP of the tibial nerve, and the CSA of GCM muscle fibers (type I, II, and total) in the G4-PDRN + ESWT and G3-ESWT groups were significantly greater than those in the other groups. Several previous studies have reported that short-term post-injury ESWT promoted increased tissue-specific expression of growth factors (8,9,15,22,23).

Other previous experimental studies have shown that such a response was also observed in injured bones, tendons, and toxin-lesioned limb muscles in animal models (15,23,26-28). In those studies, ESWT induced the upregulation of VEGF, VEGF receptor protein, placental growth factor (PGF), PGF receptor, and transforming growth factor β1. As a result, it stimulated the presence of mitotic precursor cells and angiogenesis, and promoted healing. In our study, the VEGF and PECAM-1 ratio of GCM muscle fibers in the G4-PDRN + ESWT group were significantly higher than those in the other three groups. Frey *et al.* reported that the recovery of injured muscle strength was more remarkable in the locally VEGF-treated rabbit group than in the control group by reducing connective tissue amount and increasing the number of muscle fibers (29). PECAM-1 is also known to be involved in angiogenesis, which is necessary for healing and the regeneration of nutrients and immune cells (30,31).

Our study also showed that PDRN injection could play a role in promoting recovery from atrophy caused by immobilization. According to previous studies, PDRN can promote cell growth and migration, produce extracellular matrix proteins, and decrease inflammation

Page 12 of 14

Kim et al. PDRN and shock wave on muscle atrophy.

(4,32). Furthermore, PDRN can be transported into the cell to supply pyrimidine or purine rings, which can be substrate sources of enzymes for salvage pathways. Through this process, PDRN can activate the production of nucleic acids (33). By binding to certain receptors, purine nucleosides can stimulate different transduction pathways, performing as mitogens of endothelial cells, neuroglia (34), and showing synergetic action with other growth factors (35,36). A previous study reported that PDRN stimulates VEGF expression by activating the adenosine $A_{2A}$ receptor (32). Additionally, a recent study reported that VEGF could promote healing in diabetic feet in rats by inducing angiogenesis and collagen synthesis (33). Thus, PDRN might also promote the healing of degenerative tissues or atrophied muscles, which also have poor vascularization (1,2). Based on the results that showed a significantly greater number of GCM muscle fibers (type I, II, and total) in the G4-PDRN + ESWT group than in the other three groups, we suggest that ESWT-induced VEGF expression increases the regenerative effect of PDRN.

In this study, we injected 0.7 mL of PDRN into the lateral and medial head of the GCM under ultrasound guidance. The optimal route or dosage of administration of PDRN for regenerating atrophied muscle has not yet been established. A recent study used 5.625 mg/3 mL of PDRN in patients with rotator cuff disease (37). Another study that used a rabbit model with rotator cuff disease used 0.2 mL of PDRN because rabbits' weights are approximately 5% of the weight of a typical human adult (38). We estimated the rabbit GCM muscle size and used 0.7 mL of PDRN because the size of the GCM muscle of a rabbit was approximately 3.5-fold greater than the size of its supraspinatus. To confirm the regenerative effect of PDRN in atrophied muscles, we believe that it is necessary to study the optimal route and dosage of PDRN administration.

This study has some limitations. First, this study evaluated the results at four weeks from baseline after two weeks of IC. Therefore, it is necessary to investigate the long-term effects of PDRN injections with or without ESWT on atrophied muscles. Second, we performed two sessions of ESWT using a radial-type machine at a frequency of 3 Hz, energy density of 0.1 mJ/mm$^2$, and 1,500 pulses per session. We did not evaluate the differences in the efficacy of ESWT at different frequencies, energy densities, and number of pulses. Further studies are needed to investigate a more appropriate intervention for atrophied muscles. Finally, molecular studies such as CD31 staining, collagen staining, and western blotting will be needed to reveal the mechanism of the synergistic effect of PDRN injection and ESWT on atrophied muscles.

In this study, we demonstrated that ESWT combined with PDRN injection was more effective on regenerating atrophied calf muscles in rabbit models than ESWT alone, PDRN injection alone, and injection with normal saline. The mechanism of action of this intervention was found to include a higher expression of VEGF and PECAM-1, and increases in calf circumference, GCM muscle thickness, CMAP of the tibial nerve, and CSA of the GCM muscle fibers. A strength of this study was that we evaluated the CSA of two types of muscle fibers, including type 1 and type 2, in GCM muscles.

## Acknowledgments

## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-854/rc

*Data Sharing Statement:* Available at https://atm.amegroups.com/article/view/10.21037/atm-22-854/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-854/coif). All authors report that this work was supported by the grant of the Research Institute of Medical Science, Daegu Catholic University (2021). The authors have no other conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Daegu School of Medicine (IRB No. DCIAFCR-200921-10-Y), which is the author's affiliation, in compliance with IACUC guidelines for the care and use of animals.

*Open Access Statement:* This is an Open Access article distributed in accordance with the Creative Commons

## References

1. Fujino H, Kohzuki H, Takeda I, et al. Regression of capillary network in atrophied soleus muscle induced by hindlimb unweighting. J Appl Physiol (1985) 2005;98:1407-13.

2. Dirks ML, Wall BT, Snijders T, et al. Neuromuscular electrical stimulation prevents muscle disuse atrophy during leg immobilization in humans. Acta Physiol (Oxf) 2014;210:628-41.

3. Bergouignan A, Rudwill F, Simon C, et al. Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies. J Appl Physiol (1985) 2011;111:1201-10.

4. Kim JK, Chung JY. Effectiveness of polydeoxyribonucleotide injection versus normal saline injection for treatment of chronic plantar fasciitis: a prospective randomised clinical trial. Int Orthop 2015;39:1329-34.

5. Bitto A, Polito F, Irrera N, et al. Polydeoxyribonucleotide reduces cytokine production and the severity of collagen-induced arthritis by stimulation of adenosine A($_2$A) receptor. Arthritis Rheum 2011;63:3364-71.

6. Bitto A, Galeano M, Squadrito F, et al. Polydeoxyribonucleotide improves angiogenesis and wound healing in experimental thermal injury. Crit Care Med 2008;36:1594-602.

7. Galeano M, Bitto A, Altavilla D, et al. Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse. Wound Repair Regen 2008;16:208-17.

8. Assaly R, Giuliano F, Clement P, et al. Extracorporeal Shock Waves Therapy Delivered by Aries Improves Erectile Dysfunction in Spontaneously Hypertensive Rats Through Penile Tissue Remodeling and Neovascularization. Sex Med 2019;7:441-50.

9. Murata R, Ohtori S, Ochiai N, et al. Extracorporeal shockwaves induce the expression of ATF3 and GAP-43 in rat dorsal root ganglion neurons. Auton Neurosci 2006;128:96-100.

10. Rompe JD, Decking J, Schoellner C, et al. Shock wave application for chronic plantar fasciitis in running athletes. A prospective, randomized, placebo-controlled trial. Am J Sports Med 2003;31:268-75.

11. Spacca G, Necozione S, Cacchio A. Radial shock wave therapy for lateral epicondylitis: a prospective randomised controlled single-blind study. Eura Medicophys 2005;41:17-25.

12. Martínez IM, Sempere-Rubio N, Navarro O, et al. Effectiveness of Shock Wave Therapy as a Treatment for Spasticity: A Systematic Review. Brain Sci 2020;11:15.

13. Hsu PC, Chang KV, Chiu YH, et al. Comparative Effectiveness of Botulinum Toxin Injections and Extracorporeal Shockwave Therapy for Post-Stroke Spasticity: A Systematic Review and Network Meta-Analysis. EClinicalMedicine 2021;43:101222.

14. Lee JH, Cho SH. Effect of extracorporeal shock wave therapy on denervation atrophy and function caused by sciatic nerve injury. J Phys Ther Sci 2013;25:1067-9.

15. Zissler A, Steinbacher P, Zimmermann R, et al. Extracorporeal Shock Wave Therapy Accelerates Regeneration After Acute Skeletal Muscle Injury. Am J Sports Med 2017;45:676-84.

16. Kauhanen S, von Boguslawsky K, Michelsson JE, et al. Satellite cell proliferation in rabbit hindlimb muscle following immobilization and remobilization: an immunohistochemical study using MIB 1 antibody. Acta Neuropathol 1998;95:165-70.

17. Schmitz C, Császár NB, Milz S, et al. Efficacy and safety of extracorporeal shock wave therapy for orthopedic conditions: a systematic review on studies listed in the PEDro database. Br Med Bull 2015;116:115-38.

18. Park DS, Kwon DR, Park GY, et al. Therapeutic Effect of Extracorporeal Shock Wave Therapy According to Treatment Session on Gastrocnemius Muscle Spasticity in Children With Spastic Cerebral Palsy: A Pilot Study. Ann Rehabil Med 2015;39:914-21.

19. Wu YT, Ke MJ, Chou YC, et al. Effect of radial shock wave therapy for carpal tunnel syndrome: A prospective randomized, double-blind, placebo-controlled trial. J Orthop Res 2016;34:977-84.

20. Chang KV, Chen SY, Chen WS, et al. Comparative effectiveness of focused shock wave therapy of different intensity levels and radial shock wave therapy for treating plantar fasciitis: a systematic review and network meta-analysis. Arch Phys Med Rehabil 2012;93:1259-68.

21. Park KD, Lee WY, Park MH, et al. High- versus low-energy extracorporeal shock-wave therapy for myofascial

Page 14 of 14

Kim et al. PDRN and shock wave on muscle atrophy.

pain syndrome of upper trapezius: A prospective randomized single blinded pilot study. Medicine (Baltimore) 2018;97:e11432.

22. Wang CJ, Huang HY, Pai CH. Shock wave-enhanced neovascularization at the tendon-bone junction: an experiment in dogs. J Foot Ankle Surg 2002;41:16-22.

23. 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;22:854-61.

24. Zhang D, Kearney CJ, Cheriyan T, et al. Extracorporeal shockwave-induced expression of lubricin in tendons and septa. Cell Tissue Res 2011;346:255-62.

25. Mattyasovszky SG, Langendorf EK, Ritz U, et al. Exposure to radial extracorporeal shock waves modulates viability and gene expression of human skeletal muscle cells: a controlled in vitro study. J Orthop Surg Res 2018;13:75.

26. Holfeld J, Tepeköylü C, Blunder S, et al. Low energy shock wave therapy induces angiogenesis in acute hind-limb ischemia via VEGF receptor 2 phosphorylation. PLoS One 2014;9:e103982.

27. Wang CJ, Wang FS, Yang KD. Biological effects of extracorporeal shockwave in bone healing: a study in rabbits. Arch Orthop Trauma Surg 2008;128:879-84.

28. Barnes K, Lanz O, Werre S, et al. Comparison of autogenous cancellous bone grafting and extracorporeal shock wave therapy on osteotomy healing in the tibial tuberosity advancement procedure in dogs. Radiographic densitometric evaluation. Vet Comp Orthop Traumatol 2015;28:207-14.

29. Frey SP, Jansen H, Raschke MJ, et al. VEGF improves skeletal muscle regeneration after acute trauma and reconstruction of the limb in a rabbit model. Clin Orthop Relat Res 2012;470:3607-14.

30. DeLisser HM, Christofidou-Solomidou M, Strieter RM,

et al. Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol 1997;151:671-7.

31. DiPietro LA. Angiogenesis and wound repair: when enough is enough. J Leukoc Biol 2016;100:979-84.

32. Veronesi F, Dallari D, Sabbioni G, et al. Polydeoxyribonucleotides (PDRNs) From Skin to Musculoskeletal Tissue Regeneration via Adenosine A2A Receptor Involvement. J Cell Physiol 2017;232:2299-307.

33. Altavilla D, Bitto A, Polito F, et al. Polydeoxyribonucleotide (PDRN): a safe approach to induce therapeutic angiogenesis in peripheral artery occlusive disease and in diabetic foot ulcers. Cardiovasc Hematol Agents Med Chem 2009;7:313-21.

34. Rathbone MP, Middlemiss PJ, Gysbers JW, et al. Purine nucleosides and nucleotides stimulate proliferation of a wide range of cell types. In Vitro Cell Dev Biol 1992;28A:529-36.

35. Rathbone MP, Deforge S, Deluca B, et al. Purinergic stimulation of cell division and differentiation: mechanisms and pharmacological implications. Med Hypotheses 1992;37:213-9.

36. Wang DJ, Huang NN, Heppel LA. Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. Biochem Biophys Res Commun 1990;166:251-8.

37. Yoon YC, Lee DH, Lee MY, et al. Polydeoxyribonucleotide Injection in the Treatment of Chronic Supraspinatus Tendinopathy: A Case-Controlled, Retrospective, Comparative Study With 6-Month Follow-Up. Arch Phys Med Rehabil 2017;98:874-80.

38. Kwon DR, Park GY, Lee SC. Treatment of Full-Thickness Rotator Cuff Tendon Tear Using Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Polydeoxyribonucleotides in a Rabbit Model. Stem Cells Int 2018;2018:7146384.