ORIGINAL ARTICLE

# Shoulder rotator cuff responses to extracorporeal shockwave therapy: morphological and immunohistochemical analysis

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None declared

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## ABSTRACT

***Background*** Application of extracorporeal shockwave therapy (ESWT) induces an improvement in tissue healing associated with augmented tissue perfusion. The present study aimed to investigate the responses of human rotator cuff tissue to the application of ESWT.

***Methods*** Thirty-one consecutive patients with symptomatic rotator cuff tendinopathy with complete tears were approached and enrolled in the present study. Before surgical resolution, a single treatment of focused ESWT was offered to all patients. Ten patients accepted such treatment and 21 refused ESWT. Tendon tissue biopsies were collected for evaluation using haematoxylin and eosin and characterized according to the Riley Classification. Vascular volume area (VVA) was determined semi-quantitatively and immunohistochemical (IHC) analysis included CD14, CD34, PCNA, Tenascin-C and D2-40 markers.

***Results*** Distribution of grade according to the Riley Classification with respect to study group was: Group A: Grade III ($n = 9$), Grade IV ($n = 1$); Group B: Grade III ($n = 13$), Grade IV ($n = 8$). Mean group-specific VVA analysis was 18.47% and 7.03% for Group A and Group B, respectively. IHC Grade III protein staining was significantly more prevalent in Group A compared to Group B for CD34, PCNA, Tenascin-C and D2-40 ($p < 0.05$ for all comparisons).

***Conclusions*** ESWT is associated with increased neovascularization and neolymphangiogenesis in rotator cuff tendinopathy. IHC analysis suggests an improvement in healing response in the ESWT-treated tendon.

## INTRODUCTION

Rotator cuff tears are one of the most common causes of pain and disability affecting the upper extremity. Rotator cuff tendinopathy refers to intrinsic tendon degeneration or the failure of tendon fibres at a microscopic level [1,2]. The primary goal of the treatment of this condition is to reduce or eliminate pain and, in turn, to restore function for activities of daily living [3,4]. Both nonsurgical and surgical treatment options are available. Nonsurgical treatment involves rest and activity technique modification, correction of underlying mechanical abnormalities of the shoulder such as muscle weakness and fatigue and, finally, time and patient forbearance. Hence, tendon healing is a slow process, often taking many weeks to several months. A failure to respond to the conventional methods of non-operative treatment, patient age $<$60 years, and clinically evident full-thickness rotator cuff tears are all indications for surgical treatment. A clinically significant percentage of patients have complete tears. Indeed, a study of 306 cadaveric shoulders reported a 19% incidence of full-thickness tears within the supraspinatus tendon [5].

Chronic tendinopathies are well known with respect to the decreased healing capacity of hypocellular tendons. The reported re-tear index after surgical treatment varies from 25% to 95% several months to several years after the operation [6–8]. A crucial factor for the success of reparative surgery for rotator cuff tendinopathy is the morphological 'quality' of the tendons. Cell viability, extent of tendon fibre damage as a result of prolonged inflammation, and blood and nutrient supply within the affected tissue are all important factors contributing to the success or failure of surgical treatment. Other studies have shown that a significant decrease of oxygen consumption, increased indices of apoptosis and severe metabolic disturbance all negatively impact the capacity of tendon tissue repair [9–14]. In a series of patients undergoing surgery for rotator tendinopathy, Matthews et al. demonstrated morphologically that 46% of re-tears were associated with previously-mentioned factors associated with an adverse healing response [8]. Therefore, methods to stimulate cellular synthesis and replication, ultimately rapid and robust tendon tissue repair, which could in turn add to the success rate of surgery, are needed. Various modalities have been tested to achieve this aim and include electrical and electromagnetic stimulation of the tendon [15,16]; polypeptide growth factors, which promote wound healing [17]; and osteoinductive growth factors, such as bone morphogenetic protein (BMP)-2, BMP-7 and BMP-12, transforming growth factor (TGF)-b1, TGF-b2 and TGF-b3, fibroblast growth factor (FGF), platelet-rich plasma, and various tendon substitutes [18–20].

 

The capability of extracorporeal shockwave therapy (ESWT) to induce neovascularization and hypercellularity in various tissues such as bone [21–24], tendon [25,26], skin [27–31] and myocardium [32–34] led to its investigation and application in a wide spectrum of clinical indications. ESWT has already received regulatory approval for some clinical indications, such as plantar fasciitis with or without heel spur; Achilles tendinopathy; radial epicondilopathy (tennis elbow); rotator cuff tendinopathy with or without calcification; patella tendinopathy; and greater trochanteric pain syndrome. ESWT is also commonly/empirically used as a treatment for ulnar epicondilopathy, adductor syndrome, pes anserinus syndrome and peroneal tendon syndrome. However, the mechanism of action of the favourable impact of ESWT on healing and tissue repair is not fully understood and is under investigation worldwide. The present study investigated the effects of ESWT on rotator cuff tissue in patients with complete tears undergoing operative repair with or without a single treatment of focused ESWT, aiming to determine the neo-angiogenic potential and tissue repair capability of this modality at the morphological and tissue protein (immunohistochemical) level.

## MATERIALS AND METHODS

From January 2006 to June 2009, we enrolled 31 consecutive patients [mean (SD) age, 61 (6) years] into a prospective institutional review board approved clinical study. All patients presented with chronic shoulder dysfunction. Inclusion criteria were tendinopathic rotator cuff and complete medium to large tears, which were assessed by ultrasonography. After providing their informed consent to participate in the study, patients were given the option to receive pre-operative single focused shockwave treatment (4000 impulses; energy density flux = 0.3 mJ/mm$^2$, with no anaesthesia) followed by delayed operative repair (8 weeks to 10 weeks later) or to proceed straight away to surgery without shockwave treatment. All patients consented to tendon tissue biopsy collection. Ten patients accepted pre-operative shockwave therapy (Group A), and 21 patients refused the shockwave therapy (Group B) and underwent surgery according to schedule. Routine biopsies from the edge of the torn rotator cuff (2 mm to 4 mm wide by 10 mm to 16 mm long) were collected and paraffin-embedded in tissue blocks. Patients in both groups had similar demographic characteristics and duration of symptoms; none had previous operation for rotator tendinopathy. The protocol was approved by the Institutional Ethics Committee of the Faculty of Medicine, University of Chile.

### *ESWT*

After consenting to receive ESWT, patients were scheduled to receive a single session of focused shockwave therapy as an outpatient procedure, without anaesthesia. Each patient received 4000 impulses (energy flux density: 0.3 mJ/mm$^2$). Shockwaves were applied on the affected shoulder through ultrasound gel, orientating the shockwave energy front towards the supraspinatus/infraspinatus lesion under ultrasound guidance. Orthospec (Medispec., Yehud, Israel) or Storz Duo-Lith (Karl Storz GmbH & Co. KG, Tuttlingen, Germany) were used for the generation of shockwaves. Complete treatment was administered in the recumbent position and lasted 25 minutes to 35 minutes.

### *Histological analysis*

Tissue biopsies were fixed in formalin, embedded in paraffin blocks, cut at 2 µm thickness using a Leica microtome (RM2125 RTS; Leica, Wetzlar, Germany) and placed onto Snowcoat X-tra glass slides (Leica). After positioning of the tissue onto the slides, sections were deparaffinized in xylene, rehydrated in graded alcohol and stained with haematoxylin and eosin (H&E) stains. Light microscopy (Nikon Eclipse E-200; Nikon Instruments Inc., Tokyo, Japan) was used for the standard grading of tendinopathic tissue in accordance with the Riley Classification. Three sections per patient (total of 93 slides) were stained with H&E and photographed under ×10 magnification; further analysis was performed on microphotographs. The same images were analyzed with a 10 × 10 grid (100 chambers) providing the basis for semi-quantitative analysis of vascular volume area (VVA). Only clearly defined vascular structures were taken into account and images were obtained under ×4, ×40 and ×100 magnification for vascular evaluation and semi-quantitative analysis.

### *Immunohistochemistry*

To gain an understanding of the underlying mechanisms of ESWT in rotator cuff tendinopathy with complete tear, the visualization and expression of protein markers of blood and lymph vasculature and extracellular matrix was conducted. We stained tissue sections of interest with commercially available anti-CD14 (5A3B11B5; sc-58951; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD34 (43A1; sc-65261; Santa Cruz Biotechnology), anti-PCNA (PC11; sc-53407; Santa Cruz Biotechnology), anti-Tenascin-C (2Q568; sc-73139; Santa Cruz Biotechnology) and anti-D2-40 (MA1-83884; Affinity Bioreagents, Golden, CO, USA) antibodies. Briefly, tissue sections were deparafinized in xylene, rehydrated in graded alcohol and further steps were performed in accordance with the manufacturer's instructions; three slides were stained for each marker. We obtained five photomicrographs for each marker under light microscopy (Nikon Eclipse E-200) at magnification ×40 and performed semi-quantitative image analysis using Image Pro-Plus, version 6.2 (Media Cybernetics, Inc., Bethesda, MD, USA).

### *Statistical analysis*

Statistical analysis was performed using STATA, version 11.1 (Stata Corp., College Station, TX, USA). Analysis of variance for repeated measures was performed for continuous parameters. We compared tendinopathy Grade III between treated (Group A) and untreated (Group B) study groups and tendinopathies grade III and IV within Group B. The confidence interval for each marker was set at 95%.

## RESULTS

Patients in the shockwave-treated group (Group A) reported no pain during treatment, and no adverse events related to the treatment were observed. There were neither superficial, nor

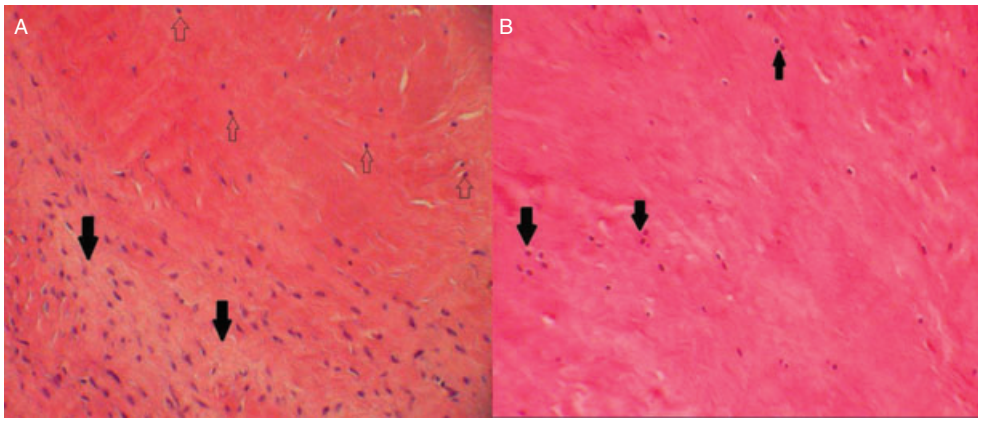


**Fig. 1** (A) Tendinopathy Grade III Riley's classification. Featured by kariopicnosis with dark and rounded nucleus (open arrows); tendon collagens appear pale and disorganized, with initial hyalinization of the tendon tissue (black arrows) [haematoxylin and eosin (H&E); ×10]. (B) Tendinopathy Grade IV Riley's classification. Depicting the absence of wavy-collagen disposition and higher tissue hyalinization. Cell nuclei appear rounded, many of them in clusters, resembling chondroid metaplasia (arrows) (H&E, ×10).

deep infections or haematomas in the shockwave-treated group. The average follow-up period for postoperative pain, time for healing of surgical wounds or functional improvement during the postoperative rehabilitative regimens was 10 months. The surgical findings identified no differences between groups and the postoperative course was similar during clinical follow-up.

### *Histological analysis*

According to the Riley Classification, tendinopathy Grade III (Fig. 1A) was found in nine cases (90%) in Group A and 13 cases (62%) in Group B. For tendinopathy Grade IV (Fig. 1B), there was one (10%) patient in Group A, and eight (38%) patients in Group B. VVA was 18.47% for Group A and 7.03% for Group B (Table 1). Taking into consideration only tendinopathy Grade III, in treated patients, VVA was 20.5% and, for same class of nontreated group subjects, it was 10.3%. For tendinopathy Grade IV (Group B), VVA was 1.6% and, in Group A, VVA was 0%. We observed two types of vascular arrangements when neo-angiogenesis was present. The first type, designated as 'diffuse neo-angiogenesis' was characterized by many non-active neo-vessels invading diffusely the hypocellular tendon; incomplete surrounding pericytes were sometimes seen associated with micro-haemorrhages in the tendon stroma (Fig. 2A). The second type, 'nodular neo-angiogenesis', was characterized by mostly active vessels with both a normal number and distribution of pericytes, and significant surrounding cellularity, as well as the synthesis of immature neo-collagens that appeared to stabilize the healing tendon; these structured vessels never demonstrated any morphological association with micro-haemorrhages. The anatomical position of these structures in the nontreated tendon was in the sub-synovial (bursae areas) region, approximating large blood vessels, and its frequency was <10% on 63 H&E slides. For ESWT-treated tendons (30 slides H&E), we observed augmented regions of 'nodular neo-angiogenesis', with the same features described above, although within deeper tissues layers (Fig. 2B–D).

**Table 1** Vascular volume areas (VVA)

| | Group A (ESWT) | | Group B (nontreated) | |
|---|---|---|---|---|
| Riley classification | Number of cases | VVA (%) | Number of cases | VVA (%) |
| Tendinopathy grade III | (nine observations) | 20.5 | (thirteen observations) | 10.3 |
| Tendinopathy grade IV | (one observation) | 0 | (eight observations) | 1.6 |
| Total | 10 | 18.5 | 21 | 7.0 |

ESWT, extracorporeal shockwave therapy.

### *Immunohistochemical analysis*

Statistical analysis indicated a small variability in the recorded data ($r^2 < 0.30$) (Tables 2 and 3); however, statistically significant differences in the expression of CD34, PCNA, Tenascin-C and D2-40 were evident between tendinopathy Grade III in shockwave-treated versus nontreated groups ($p < 0.05$) (Table 2). We also compared the same markers within group B, which had 13 Grade III tendinopathies and eight Grade IV tendinopathies. Statistical analysis showed a higher expression of CD14, CD34, Tenascin-C and PCNA for Grade III compared to Grade IV ($p < 0.05$) (Table 3).

## DISCUSSION

The number of patients suffering from chronic shoulder tendinopathy is increasing worldwide. A significant percentage of this patient population has complete tear(s) of the tendon structures [5,6]. The goal of the treatment of chronic tendinopathy is a functional improvement in terms of arm movement, recovery of strength and a return of the patient to everyday activities. If there is a failure to respond to nonsurgical treatment after 6 weeks to 8 weeks of therapy, surgical options are recommended.

The anatomical and physiological features of human shoulder rotator cuff are unique [35–37] and have been shown to comprise

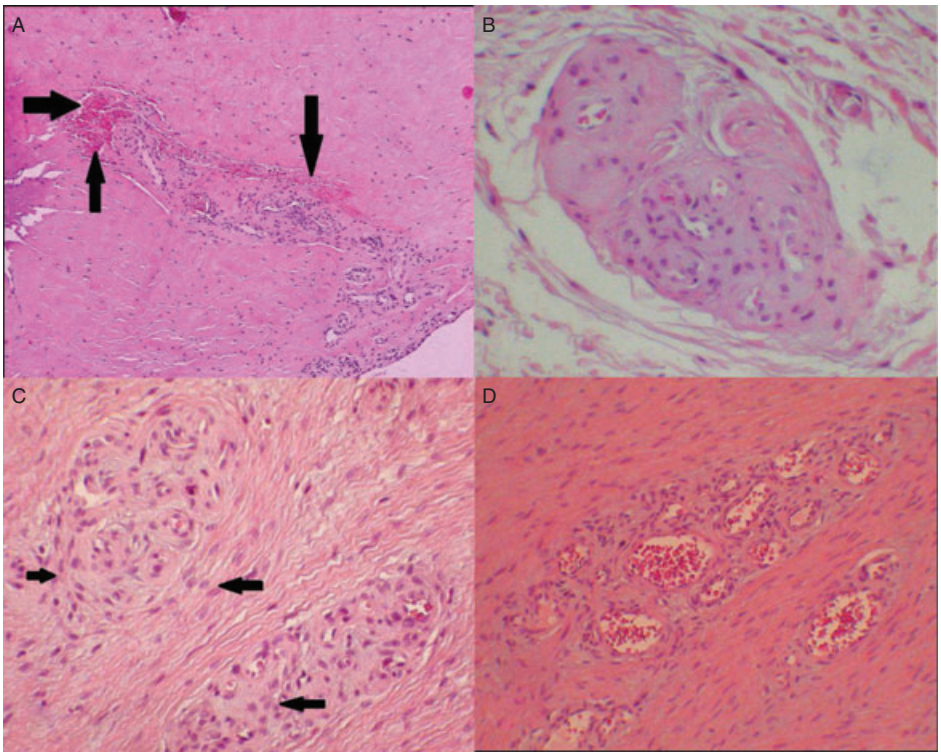


**Fig. 2** (A) Tendinopathy Grade III nontreated, depicting invasive 'diffuse neo-angiogenesis' nascent from sub-synovial normal vessels. Along the entire neovascular structure, it is possible to identify haemorrhagic foci, without an acute inflammatory response (arrows) [haematoxylin and eosin (H&E); ×4]. (B) Tendinopathy Grade III nontreated, showing sub-synovial (bursae areas) 'nodular neo-angiogenesis' depicting active vessels well-surrounded by perycites. Amorphous collagens inside the nodule appear to stabilize the node itself (H&E, ×10 and zoom). (C) Tendinopathy Grade III shockwave-treated, depicting details of the nodular vascular structure, characterized by cobblestone cells in the matrix of the nodule, with many of them migrating into native tendon collagens (arrows) (H&E, ×10 and zoom). (D) Tendinopathy Grade III shockwave-treated, demonstrating 'nodular neoangiogenesis' on deep tendon tissue. Many active blood vessels with a well-formed wall, immersed in immature collagens. Absence of micro-haemorrhagic foci (H&E, ×10).

**Table 2** Distribution of data comparing both groups for cases defined as tendinopathies grade III

| Marker | $r^2$ | Model | *p*-value group |
|---|---|---|---|
| CD14 | 0.03 | 0.664 | 0.1800 |
| CD34 | 0.10 | 0.060 | 0.0116* |
| PCNA | 0.10 | 0.124 | 0.0065* |
| Tenascin-C | 0.30 | 0.000 | 0.0000* |
| D2-40 | 0.09 | 0.098 | 0.0038* |

*Statistically significant.

**Table 3** Distribution of data for Group B comparing tendinopathy III versus IV

| Marker | $r^2$ | Model | *p*-value grade |
|---|---|---|---|
| CD14 | 0.05 | 0.3913 | 0.0276* |
| CD34 | 0.14 | 0.0125 | 0.0002* |
| PCNA | 0.16 | 0.0634 | 0.0068* |
| Tenascin-C | 0.19 | 0.0007 | 0.0004* |
| D2-40 | 0.06 | 0.3009 | 0.0532 |

*Statistically significant.

critical determinants of the successful healing of partial- and full-thickness tears after surgical intervention. Matthews et al. reviewed a series of cases of re-tears at a histological level and many of the tears demonstrated profound distortion of the tendon tissue [8]. Therefore, augmentation of the 'quality' of tendinopathic tissue is imperative for the later success of re-operative surgery.

Morphological analysis focusing on vascular areas (comprising spontaneous neo-angiogenesis) demonstrated a common 'diffuse neo-angiogenesis' (Fig. 2A), and the existence of 'nodular neo-angiogenesis' in six of eight patients with tendinopathies Grade III in Group B (Fig. 2B). Interestingly, the anatomical localization of these vascular findings was sub-synovial, and near larger blood vessels. In the shockwave-treated group, we also observed similar findings, although with a noted difference in the histological location of angiogenic foci, which were positioned in deeper tissue layers (Fig. 2C, D). We hypothesize that 'nodular' vascular arrangements represent postnatal vasculogenesis, as described previously [38,39]. This may serve as the physiological basis for the further study of mechanical transduction mediated by ESWT with the purpose of stimulating tendon repair in the setting

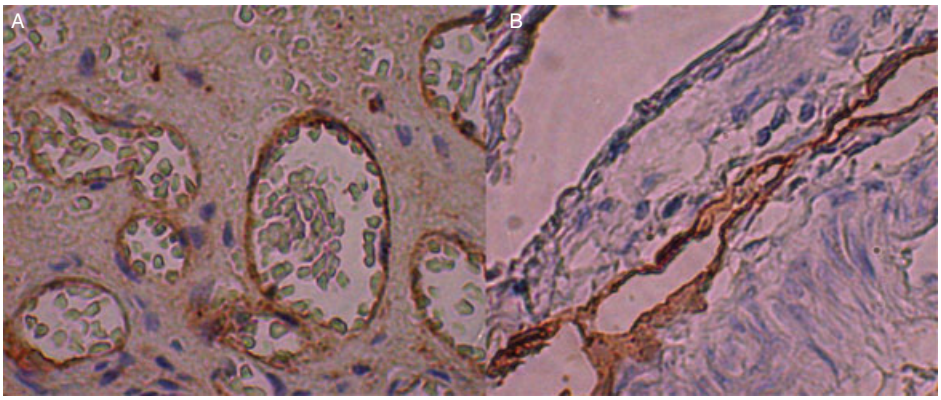


**Fig. 3** (A) Immunohistochemical (IHC) analysis for CD34. Tendinopathy Grade III shockwave-treated, neo-angiogenesis foci showing CD34 expression that appears adequate and related to neo-endothelial development (×40). (B) IHC for D2-40. Tendinopathy Grade III shockwave-treated, demonstrating the development of lymphatic vessels, contiguous to vascular structures (×40).

of chronic tendinopathy and complete tendon disruption. Tei et al. showed that CD34+ mononuclear cells derived from bone marrow and infused into the disease tissue were able to incorporate into neo-angiogeneic foci, and also participated in ligament tissue repair [40]. In the present study, we observed that neo-angio/vasculogenic foci in treated-patients were more frequent, demonstrating augmented cellularity and higher expression of CD34, PCNA and Tenascin-C, indicative of active re-vascularization and a tendency toward tendon tissue repair (Fig. 3A). In the published literature, our findings are supported by studies such as that of Aicher et al., who showed an enhanced recruitment of endothelial progenitor cells in chronic hind limb ischemia after pre-treatment of external shockwaves, along with increased vasculogenesis and tissue perfusion in the pre-treated limb [41]. More recently, Sun et al. summarized the molecular and cellular mechanisms demonstrated for shockwave applications related to pro-angiogenic effects, indicating that shockwave treatment may improve tissue perfusion by an increased recruitment of circulating endothelial progenitors cells in ischaemic tissues [42].

VVA was a parameter defined for human rotator cuff by Brook et al., with a rank ranging from 1.5% to 2% (15 mm to 30 mm from tendon insertion) [36]. Tendinopathies Grade III in shockwave-treated patients showed an augmented frequency of active vascular fields and no response in tendinopathy Grade IV. This information should be interpreted with caution because the shockwave-treated group had only one patient with tendinopathy Grade 4. In Group B (surgery alone), tendinopathies Grade III showed VVA ranking 10.3% and tendinopathies Grade IV ranked 1.6%. This semi-quantitative vascular analysis demonstrated not only improved blood supply in shockwave-treated tendons, but also spontaneous tissue healing and repair in Grade III Group B (non-shockwave, surgery only). However, 'diffuse neo-angiogenesis' foci (Group B/Grade III) (Fig. 2A) also showed anomalous pericyte envelopes with subsequent haemorrhagic areas in an marked percentage of cases. This highlights the lack of an inflammatory response to blood cell extravasations, and we do not have a plausible explanation for these histological findings. In summary, our observations from the histological analysis are indicative of improved vascularization in ESWT-treated rotator cuff tendons with complete tears.

The immunohistochemical findings suggest that tendinopathies Grade III in Group B display improved indices for CD14, CD34, Tenascin-C and PCNA than Grade IV for the same group (Table 3) ($p < 0.05$). As expected, these findings indicate an improved tendon tissue 'quality' in Grade III patients and an increased responsiveness to biological inducers of healing response. At the same time, we should expect low reactivity in tendinopathies Grade IV, which is reflected by VVA and concordant with previous results [8]. In tendinopathies Grade III for both Group A and Group B, markers CD34, Tenascin-C, PCNA and D2-40 ($p < 0.05$) were significantly differentially expressed. More vascularization (CD34) supports the VVA results, as well as the hypothesis of improved tissue repair (Tenascin-C and PCNA results).

Interestingly, D2-40, a marker of lymphatic endothelium (Fig. 3B), was more abundant in shockwave-treated patients, and indicative of stimulated lymphangiogenesis ($p < 0.0038$). This interesting observation provides insight into the positive results of ESWT in calcified shoulder tendinopathies. We observed a treatment response (i.e. modulation in the mobilization of calcified deposits after ESWT) comprising developing 'calcium lines' in sub-synovial areas from lateral to medial, as well as the upper one-third portion of the treated rotator cuff. Recent animal studies report shockwave-induced lymphangiogenesis [43,44].

We do recognize that there are limitations to the present study. Statistical analysis showed a small variability in the data, which was attributable to the small number of patients studied. Therefore, repeating the study with a larger patient population may lead to an adequately powered statistical analysis of the treatment effects of shockwaves for this clinical indication. Nevertheless, for the first time, through preliminary morphological and immunohistochemical assessment, we report hypothesis-generating data providing a biological basis for the shockwave treatment of shoulder rotator cuff tendinopathies, as evident through induced neovasculogenesis and angiogenesis, as well as the stimulated development of lymph vessels inside diseased tendon tissues.

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