Ultrasound in Med. & Biol., Vol. ■, No. ■, pp. 1–12, 2014

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2013.12.003

● *Original Contribution*

SOFT-FOCUSED EXTRACORPOREAL SHOCK WAVES INCREASE THE EXPRESSION OF TENDON-SPECIFIC MARKERS AND THE RELEASE OF ANTI-INFLAMMATORY CYTOKINES IN AN ADHERENT CULTURE MODEL OF PRIMARY HUMAN TENDON CELLS

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(*Received* 17 *June* 2013; *revised* 26 *September* 2013; *in final form* 5 *December* 2013)

Abstract—Focused extracorporeal shock waves have been found to upregulate the expression of collagen and to initiate cell proliferation in healthy tenocytes and to positively affect the metabolism of tendons, promoting the healing process. Recently, soft-focused extracorporeal shock waves have also been found to have a significant effect on tissue regeneration. However, very few *in vitro* reports have dealt with the application of this type of shock wave to cells, and in particular, no previous studies have investigated the response of tendon cells to this impulse. We devised an original model to investigate the *in vitro* effects of soft-focused shock waves on a heterogeneous population of human resident tendon cells in adherent monolayer culture. Our results indicate that soft-focused extracorporeal shock wave treatment (0.17 mJ/mm^2) is able to induce positive modulation of cell viability, proliferation and tendon-specific marker expression, as well as release of anti-inflammatory cytokines. This could prefigure a new rationale for routine employment of soft-focused shock waves to treat the failed healing status that distinguishes tendinopathies. (E-mail: laura.degirolamo@grupposandonato.it) © **2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Soft-focused extracorporeal shock waves, Tendon cells, Tendinopathy, Cell proliferation, Transforming growth factor β, Vascular endothelial growth factor, Cytokines, Tendon-specific markers.**

INTRODUCTION

Tendinopathies are common pathologies, particularly among athletes, and represent about 45% of all musculoskeletal injuries (Maffulli and Kader 2002). Despite progress in the treatment of tendinopathies, several aspects related to the complex tendon pathophysiology remain unclear (Del Buono et al. 2011). There is still debate regarding the true role of inflammatory insult and overload in the activation of the processes that gradually produce degenerative changes in tendon structure as a result of qualitative and quantitative alteration of tenocytes (Abate et al. 2009; Cook and Purdman 2009; Fredberg and Stengaard-Pedersen 2008).

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As recently reported, along with tenocytes (up to 90%–95%) and a limited number of chondrocytes and endothelial cells, human tendons are also composed of tendon stem/progenitor cells (TSPCs) that have universal stem cell characteristics such as clonogenicity, multipotency and self-renewal capacity (Bi et al. 2007). They also help maintain the homeostasis of the tendon (Bi et al. 2007).

In the past, several conventional conservative approaches to the treatment of tendinopathies have been evaluated (Andreas and Murrell 2008), including extracorporeal shock waves (ESWs) (Notarnicola and Moretti 2012). Shock waves used in medical practice are non-linear, single, sonic pulses with a broad frequency spectrum ranging from 16 Hz to 20 MHz. They are characterized by rapid (<10 ns) and short (<10 μs) fluctuations of positive acoustic energy (up to 10–100 MPa) followed by a low tensile phase, near

10%–20% of the positive pressure peak (Ogden et al. 2001). These phenomena act on living tissues through specific pathways of mechanotransduction, affecting cell membrane polarization, triggering free radical formation and modulating gene expression and growth factor production (Wang et al. 2002). In healthy tenocytes, shock waves have been reported to upregulate the expression of collagen and to initiate cell proliferation (Vetrano et al. 2011), whereas in human tenocytes derived from pathologic tendons, decreases in collagen type I and scleraxis have been observed (Leone et al. 2012). In addition, different animal models have illustrated that ESWs affect local blood flow and metabolism of the tendon and promote the healing process by increasing the expression of typical growth factors (transforming growth factor β_1 [TGFβ1], insulin-like growth factor 1 [IGF1]) (Chen et al. 2004), as well as the synthesis and organization of collagen fibers (Ohran et al. 2004). Moreover, one of the most interesting aspects of the effect of shock waves on tendons is that the acoustic impulse lowers the expression of matrix metalloproteinases (MMPs) and pro-inflammatory interleukins (ILs) (Han et al. 2009), which are known to have a role in the pathogenesis of tendinopathies.

The dose-related response of the cells to shock waves seems to be closely related to the type of generator as well as to different energy settings (Martini et al. 2006). *In vivo* experiments on rabbit Achilles tendon revealed histopathological changes that varied from an inflammatory peritendinous reaction at lower energy flux density (EFD) values to capillary disruption, erythrocyte extravasation, necrosis of the tendon fibers and fibroblast proliferation at higher EFD values (Rompe et al. 1998). Again, in a model of rat tenocytes, the best results in terms of proliferation and collagen synthesis were observed at lower EFD values and lower numbers of shocks, together with an immediate and transient increase in the mediator nitric oxide (NO) (Chao et al. 2008).

The shock wave treatment outcome could be also influenced by the characteristics of the focus. Recently, soft-focused shock waves have been reported to have a significant effect on tissue regeneration (Kuo et al. 2009). The peculiarity of soft-focused shock waves lies in the possibility of delivering energy to a larger area while the temporal feature of the impulse remains unvaried. Because of this feature, soft-focused shock waves are especially suitable for *in vitro* experiments, particularly in cells adherent to a culture plate.

On the basis of these observations, we devised an original model to investigate the *in vitro* effects of soft-focused shock waves on a heterogeneous population of human resident tendon cells (TCs) in adherent monolayer culture. For our purpose, we used an electrohydraulic device in which the shock waves are produced by the high-voltage discharge of an electrode placed in the water-containing compartment. The primary shock wave front is conveyed by a parabolic reflector in a second, almost parallel, large ovoid focus (soft focus) (Mittermayr et al. 2011). The probe (OP 155 applicator) generating the shock waves is coupled to a patented water bath for the treatment of cell cultures.

After treatments, TC viability, DNA content, specific tenogenic gene expression, release of anti- and pro-inflammatory cytokines and nitric oxide production were evaluated. All experiments were performed on seven different cell populations isolated from small portions of healthy semitendinosus and gracilis tendons of seven patients who had undergone anterior cruciate ligament (ACL) reconstruction. To our knowledge, this is the first experiment of its kind to be reported, and includes a description of the effects of soft-focused shock waves on cytokine release.

METHODS

Tendon cell isolation and culture expansion

All procedures were carried out with institutional review board approval. Discarded fragments of semitendinosus and gracilis tendons were collected from seven healthy young donors (mean age = 29 ± 7 y) who underwent ACL reconstruction with autologous hamstrings at our hospital. All patients gave written consent to the procedure. To isolate TCs, we minced and enzymatically digested the tendon tissue with 0.3% type I collagenase (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with continuous agitation for 15 h at 37°C. The isolated nucleated cells were then cultured at 5×10^3 cells/cm^2 in complete medium consisting of DMEM high glucose, 10% fetal bovine serum (FBS, Sigma-Aldrich), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine (all from Life Technologies, Carlsbad, CA, USA) and supplemented with 5 ng/mL basic fibroblast growth factor (b-FGF, Peprotech, NJ, USA). Cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2; the culture medium was changed every 3 d. The TCs remained quiescent for about 5 d before starting to proliferate rapidly. When TCs reached 80%–90% of confluence, they were detached by incubation with 0.5% trypsin/0.2% EDTA (Sigma-Aldrich) and then expanded at a density of 3×10^3 cells/cm^2. Cells from passages 2 to 4 (P2–P4) were used for the experiments.

In vitro extracorporeal shock wave treatment

To allow complete treatment of the entire cell culture, 4×10^5 TCs were plated onto a limited area of

the T25 flask that could be reached by the shock wave impulses. For this purpose, the culture flasks were kept in an inclined position during culture for 3 d before treatment. Immediately before treatment with ESWs, the culture flasks were completely filled with DMEM to prevent air bubbles from blocking the shock wave impulses, and then the flasks were immersed in a patented system for ESW cell treatment (Holfeld et al. 2009). This system consists of a Plexiglas box containing degassed water to avoid cavitation phenomena and a heater plate to avoid thermic shock to the cells during treatment (water at 37°C). On the anterior wall of the box is a rounded porthole covered by a silicone membrane to which the shock wave applicator is applied. Standard ultrasound transmission gel is used to convey the acoustic energy to the submerged flask containing the cells. To hold the cell culture flask in place, a slotted bar, flask holder and locking screw are used to position the flask at the selected distance from the shock wave source. Finally, a wedge-shaped absorber on the back wall is used to prevent shock waves from being reflected and causing a disturbance.

The electrohydraulic shock wave device was equipped with a soft-focused applicator (OP155-Orthogold 100, MTS Europe, Konstanz, Germany). On the basis of preliminary tests to ascertain the optimal EFD in terms of cell viability and proliferation (data not shown) and on the basis of the energy flux we use in clinical practice to treat tendinopathies, the cells were treated at an EFD of 0.17 mJ/mm^2 (measurement of the manufacturer in accordance with International Electrochemical Commission procedures) corresponding to a peak positive focal pressure of 27.5 MPa (275 bar) with a tensile value of the focal pressure of 6.6 MPa (66 bar). The shock wave rise time and pulse width were respectively 162 and 505 ns (data from the manufacturer). The total energy administered to cells for each treatment was 3550 mJ.

The spatial dimension of the ovoid therapeutic focus (@ -6 dB) measured 76.5 mm in length and 17.3 mm in diameter. To ensure optimal treatment in relation to the focal dimensions, the flasks were placed 5 cm from the probe. Each cell population was treated with 1000 impulses at a pulse frequency of 3 Hz/s.

At the end of treatment, DMEM was removed and replaced with fresh complete medium; culture flasks were then incubated in a normal horizontal position for 14 d. Untreated TCs were cultured in complete medium and used as controls. The experimental design is illustrated in Figure 1.

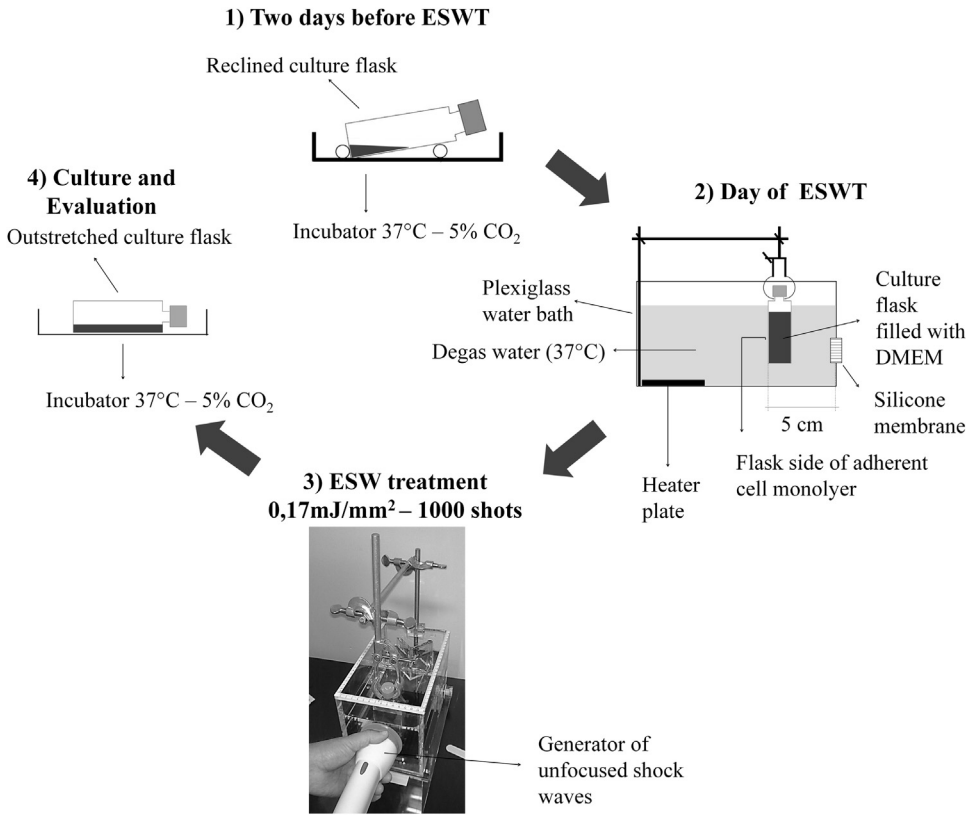

Fig. 1. Experimental scheme representing the *in vitro* shock wave treatment of adherent monolayer cell culture. DMEM = Dulbecco's modified Eagle medium, ESWT = extracorporeal shock wave treatment.

Viability and proliferation assay

At 7 and 10 d after treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to both treated and untreated cells at a final concentration of 0.5 mg/mL, and the mixtures were incubated for 4 h at 37°C. The resulting formazan precipitates were then solubilized using 100% dimethyl sulfoxide, and absorbance was read at 570 nm (VictorX3 microplate, Perkin Elmer, Waltham MA, USA) (Kingham et al. 2007). At the same time points, treated and control cells were analyzed for DNA content (Triton X-100 0.1% in ddH_2O as lysis buffer) using the CyQUANT Cell Proliferation Assay Kit (Invitrogen, Paisley, UK); fluorescence was read at 520 nm (excitation $\lambda = 480$ nm) (VictorX3 microplate).

Cell apoptosis analysis by annexin V-FITC and propidium iodide

Apoptosis induced by shock wave treatment was analyzed by flow cytometry using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (Sigma-Aldrich) 2 and 7 d after ESW treatment (ESWT). In brief, 4.0×10^5 cells were trypsinized, washed with phosphate-buffered saline and resuspended with 500 μL of a specific binding buffer containing 10 μL of PI and 5 μL of annexin V-FITC (Kuypers et al. 1996; Pigault et al. 1994). After exactly 10 min of incubation in the dark at room temperature, the cells were analyzed for annexin V and PI staining by flow cytometry. Each experiment was run in triplicate. The excitation wavelength was 488 nm; the emitted green fluorescence of annexin V (FL-1) and red fluorescence of PI (FL-2) were collected using 525- and 575-nm bandpass filters, respectively. Early apoptosis and late apoptosis/necrosis were expressed as the percentage of annexin V+/PI− and annexin V+/PI + cells.

RNA extraction, reverse transcription and real-time polymerase chain reaction

Total RNA was isolated from untreated and treated cells 1, 2, 4 and 7 d after ESWT using the RNeasy Mini Kit (Qiagen, Duesseldorf, Germany) and quantified with a spectrophotometer (Nanodrop, Thermo Scientific, Rockford, IL, USA). One hundred nanograms of RNA was reverse-transcripted to cDNA employing the iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA). The final volume of 20 μL included a 5× reaction mixture containing oligo (dT), random hexamer primers and reverse transcriptase pre-blended with RNase inhibitor. The reaction mixture was incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C.

Ten nanograms of cDNA was used as a template for real-time polymerase chain reaction (PCR), performed using a Rotor Gene RG3000 system (Qiagen). The PCR mixture included TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression probes (Life Technologies, Grand Island, NY, USA) in a final volume of 20 μL. Amplification and real-time data acquisition were performed using the following cycle conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The genes analyzed were: scleraxis (SCX) (Hs03054634_g1), type I collagen (COL1 A1) (Hs01076777_m1) and type III collagen (COL3 A1) (Hs00943809_m1). The fold change in expression of the different genes in control and treated cells was normalized on the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1).

Cytokines, MMPs and VEGF determination in conditioned medium

The cumulative levels of soluble IL-1β, IL-6, IL-10, tumor necrosis factor α (TNFα), TGFβ2 and vascular endothelial growth factor (VEGF) in the culture medium were determined 0, 24 and 48 h after ESWT using commercially available assays according to the manufacturer's instructions (R&D System, Minneapolis, MN, USA). For VEGF detection, the sensitivity of the assay was 5 pg/mL, and the intra- and inter-assay coefficients of variation were 6.6% and 6.7%, respectively. For IL-1β detection, the sensitivity of the assay was <1 pg/mL, and the intra- and interassay coefficients of variation were 2.8% and 4.1%, respectively. For IL-6 detection, the sensitivity of the assay was 2 pg/mL, and the intra- and interassay coefficients of variation were 5.8% and 3.1%, respectively. For IL-10 detection, the sensitivity of the assay was <0.5 pg/mL, and the intra- and interassay coefficients of variation were 6.6% and 8.1%, respectively. For TNFα detection, the sensitivity of the assay was 1.6 pg/mL, and the intra- and interassay coefficients of variation were 5.0% and 7.3%, respectively. For TGFβ2 detection, the sensitivity of the assay was 2 pg/mL, and the intra- and interassay coefficients of variation were 2.7% and 4.3%, respectively.

Nitric oxide quantification

At 2, 24 and 48 h after ESWT, the culture medium of treated and untreated cells was collected to evaluate cumulative NO synthesis. Nitrite (NO_2^-), the stable end product of NO, was measured using modified Greiss reagent (Sigma-Aldrich). Greiss reagent was added in a 1:1 ratio to the collected medium in a 96-well plate, then read at 550 nm with the spectrophotometer (VictorX3 microplate).

Statistical analysis

Statistical analysis was performed with GraphPad Prism Version 5.0 software (GraphPad Software, LaJolla,

CA, USA). All values are expressed as means ± standard deviations. Normally distributed values were assayed with the Kolmogorov-Smirnov normality test, whereas a one-way analysis of variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. Paired comparisons were performed using a two-tailed *t*-test. In the case of non-normally distributed values, repeated measures were compared with the Kruskal-Wallis test with Dunn's correction. Correlation analysis was performed with the two-tailed Pearson correlation test (Spearman's test for non-normally distributed values); the same test was used to evaluate the correlation between the trends of these parameters across the time points. The significance level was set at 0.05.

RESULTS

ESWT induced an increase in TC viability and proliferation

The cell culture system that we adopted allowed a reduction in the cell growth surface in the flask, so that about 80% of tendon cells directly received the shock waves. Immediately after treatment, very small void areas were observed close to the center of the focus region because of the detachment of a few cells (Fig. 2a). Nevertheless, 24 h later, the cell monolayer was completely restored (Fig. 2b). Indeed, fluorescence-activated cell sorting (FACS) analysis performed on ESW-treated cells at both 2 (Fig. 3a, b) and 7 d after treatment (Fig. 3c, d) revealed apoptosis and necrosis rates similar to those of untreated cells, indicating that the ESWT was not cytotoxic. At day 7, cell viability significantly increased with respect to the untreated cells (+36%, $p < 0.001$), and this increase was maintained even after 14 d (+26%, $p < 0.001$) (n = 7) (Fig. 4a). At the same time points, treated TCs also had a higher DNA content compared with untreated TCs (+36% and +58% at 7 and 14 d, respectively), although these differences were not statistically significant (n = 7) (Fig. 4b).

ESWT enhanced scleraxis and type I collagen gene expression

To evaluate the possible influence of ESWT on tendon-specific gene expression, mRNA levels of SCX, COL1 A1 and COL3 A1 were analyzed 1, 2, 4 and 7 d after treatment (n = 5). The day after treatment, a statistically significant increase in SCX expression was observed in ESW-treated cells as compared with untreated cells (+52%, $p < 0.05$) (Fig. 5a), although at subsequent time points, SCX transcription progressively decreased. On the other hand, COL1 A1 expression was upregulated later: indeed, 4 d after ESWT, TCs had higher levels of COL1 A1 (+56%) with respect to untreated cells, but because of the high interdonor variability, this difference was not statistically significant (Fig. 5b). Moreover, expression of COL3 A1 in treated TCs was similar to that in untreated cells at all time points (Fig. 5c), and the ratio of COL3 A1 to COL1 A1 expression did not change significantly in treated TCs (Fig. 5d).

Effect of ESWT on cytokines, growth factors and MMP release

The levels of pro- and anti-inflammatory cytokines, growth factors and matrix metalloproteinases released by TCs in the conditioned medium were evaluated 0, 1, 2 and 7 d after ESWT (n = 6). The release of IL-1β was significantly higher (increased by 571%, $p < 0.001$) in treated cells 1 d after ESWT (Fig. 6a). IL-1β production progressively decreased over time, even if statistically higher levels were still present at 7 d in treated cells compared with untreated cells ($p < 0.001$). However, at all time points, the absolute values of IL-1β were very low. On the contrary, TNFα levels were not significantly affected by ESWs at any time points (Fig. 6b). The release of IL-6 and IL-10 was also significantly higher in treated cells

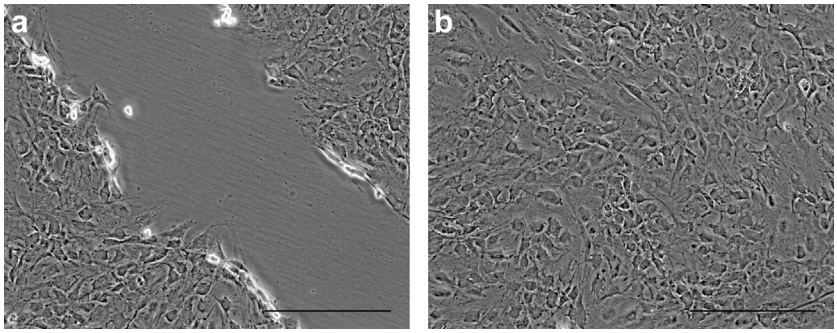

Fig. 2. Morphologic appearance of tendon cells immediately after extracorporeal shock wave treatment (a) and the next day (b). Extracorporeal shock wave treatment initially caused cellular detachment, but 24 h later the monolayer was completely restored (optical microscopy, 10×, bar = 200 μm). The shape of the void area is unrelated to the focus shape as only a few cells detached during treatment.

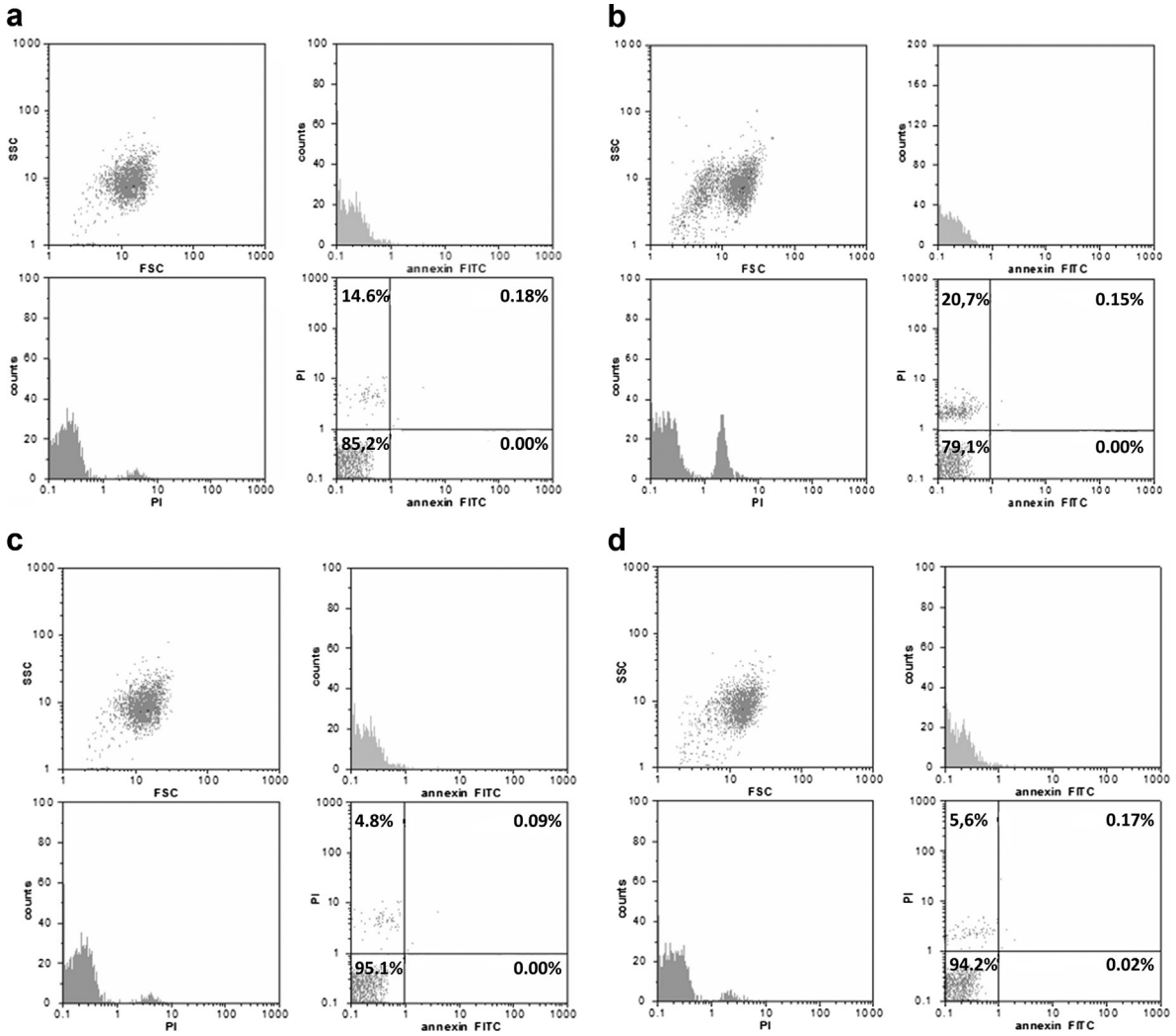

Fig. 3. Apoptosis after extracorporeal shock wave treatment. No apoptotic events were provoked by the treatment, as illustrated by the comparable rates of apoptosis and necrosis for untreated (a, c) and treated (b, d) cells at day 2 (a, b) and day 7 (c, d). SSC = side scatter, FSC = forward scatter, FITC = fluorescein isothiocyanate, PI = propidium iodide.

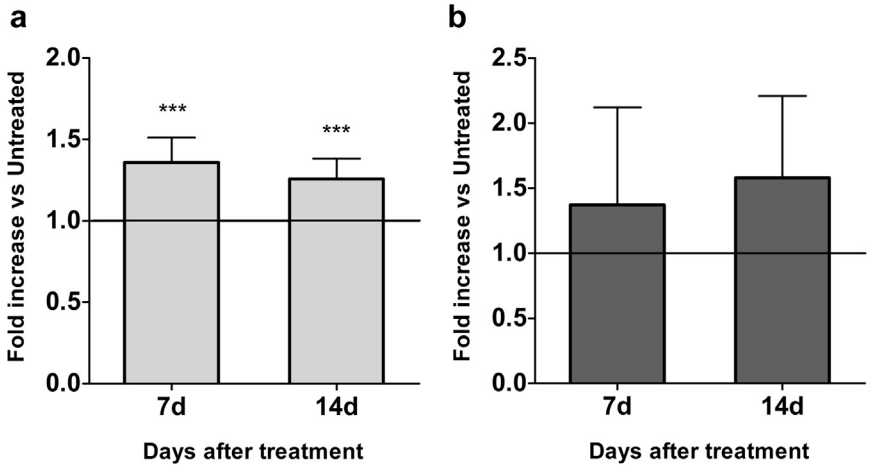

Fig. 4. Effect of extracorporeal shock wave treatment on cell viability (a) and DNA content (b) of tendon cells s after 7 and 14 d. Values are indicated as the fold increase with respect to untreated cells represented by a fixed line set at 1; data are expressed as means ± standard deviations. ***$p < 0.001$, extracorporeal shock wave-treated cells versus untreated cells, n = 7.

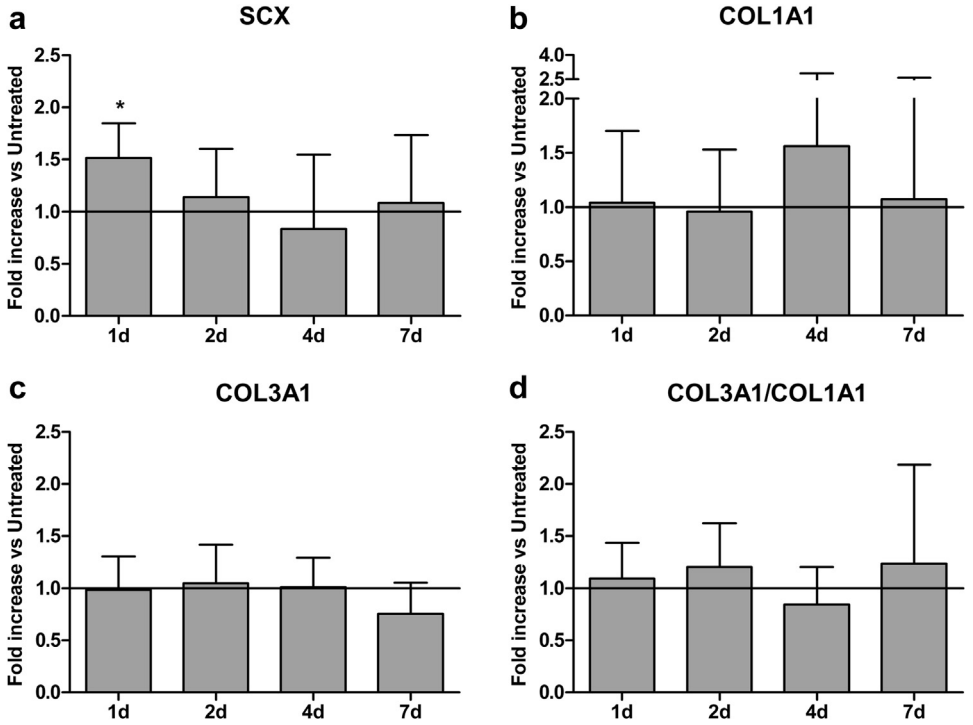

Fig. 5. Effect of exposure to extracorporeal shock waves on expression of scleraxis (SCX) (a), collagen type I (COL1 A1) (b) and collagen type III (COL3 A1) (c) and on the ratio of COL3 A1 expression to COL1 A1 expression (d), determined by quantitative real-time polymerase chain reaction at days 1, 2, 4 and 7 after treatment. Values are the fold increase in $\Delta\Delta C_t$ with respect to untreated cells represented by a fixed line set at 1, after normalization on housekeeping GAPDH gene expression. Significant modulation in gene expression was observed only for SCX, 1 d after treatment. Data are expressed as means ± standard deviations. *$p < 0.05$, extracorporeal shock wave-treated cells versus untreated cells, n = 7.

than in untreated cells. In particular, treated TCs released a significantly larger amount of IL-6 starting from day 1 after treatment (+504%, $p < 0.001$) and reaching a peak at day 2 (+641%, $p < 0.001$) (Fig. 6c). Similarly, IL-10 production significantly increased after ESWT at all time points; in particular, the highest levels were observed in treated TCs at day 2 (+471%, $p < 0.001$) (Fig. 6d). Moreover, strong and significant release of TGFβ and VEGF was observed in the culture medium of treated TCs. Indeed, TGFβ release increased 11-, 18- and 8-fold over the levels in untreated cells at days 1, 2 and 7, respectively ($p < 0.001$) (Fig. 6e). VEGF production was even more evident and was 64-, 92- and 68-fold higher in the culture medium of treated TCs 1, 2 and 7 d after treatment, respectively ($p < 0.001$) (Fig. 6f).

There was no significant difference in release of MMP-3 and MMP-13 between treated and untreated cells at any time point (Fig. 7a, b).

Nitrite evaluation

Cumulative NO production was assessed in the TC culture medium 2, 24 and 48 h after treatment. Two hours after treatment, the level of NO was undetectable in both treated and untreated medium (<0.46 μM, which corresponded to the lowest point on the standard curve). Then, 48 h after treatment, the cumulative release of NO was significantly lower in treated cells than in untreated cells ($p < 0.05$) (Table 1).

DISCUSSION

Our *in vitro* study reveals for the first time the effects of soft-focused ESWT (0.17 mJ/mm^2) in a model of healthy human adherent tendon cells. The main findings of this study are that ESWT positively influences cell viability and proliferation of TCs and modulates gene expression of tendon-specific markers, such as scleraxis, as well as the release of anti-inflammatory cytokines and the growth factors TGFβ and VEGF.

A single *in vitro* report dealt with the application of soft-focused shock waves on cells (Sansone et al. 2012), and no previous studies, to our knowledge, investigated the response of tendon cells to this kind of impulse. Preclinical studies have reported that soft-focused shock waves regulated the expression of chemokines (CXCL1, CXCL2, CXCL5), cytokines (IL-1β, IL-6, G-CSF, VEGF-A), matrix metalloproteinases (MMP-3, MMP-9, MMP-13) and specific genes involved in the angiogenic pathway in a murine model of skin isografts, indicating a pro-angiogenic effect associated with the delay of

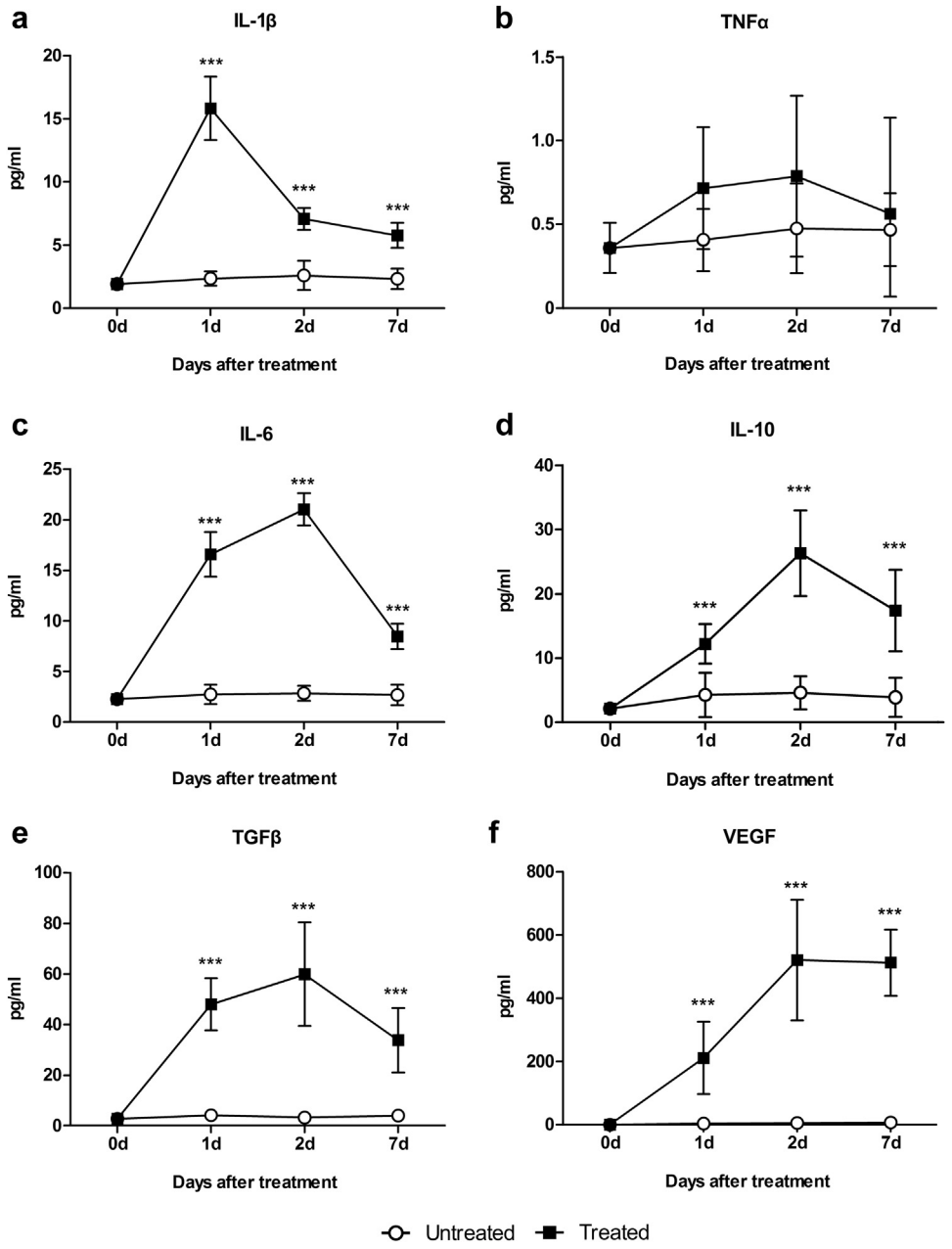

Fig. 6. Release of cytokines and growth factors 0, 1, 2 and 7 d after exposure to extracorporeal shock waves. Tumor necrosis factor α (TNFα) (a) was not affected by extracorporeal shock wave treatment, whereas the shock waves significantly increased the release of interleukin (IL)-1β (b), IL-6 (c), IL-10 (d), transforming growth factor β (TGFβ) (e) and vascular endothelial growth factor (VEGF) (f) in the culture medium compared with untreated cells.***$p < 0.001$, extracorporeal shock wave-treated cells versus untreated cells, n = 7.

inflammatory response (Stojadinovic et al. 2008). Further, in a murine model of full-thickness burn injury, soft-focused shock wave treatment reduced the inflammatory infiltrate and suppressed the expression of cytokines and MMPs (Davis et al. 2009), whereas in chronic wounds, this treatment increased blood perfusion and tissue regeneration, enhancing the expression of VEGF, endothelial nitric oxide synthase and proliferating cell nuclear antigen in fibroblasts (Kuo et al. 2009). Soft-focused shock waves have also been found to be able to minimize the extent of ischemic tissue necrosis and to induce angiogenesis in ischemic heart failure (Mittermayr et al. 2011; Zimpfer et al. 2009). Finally, other studies have investigated the protective effect of soft-focused shock waves on bone mass in experimental models of osteoporosis (Van der Jagt et al. 2009). With

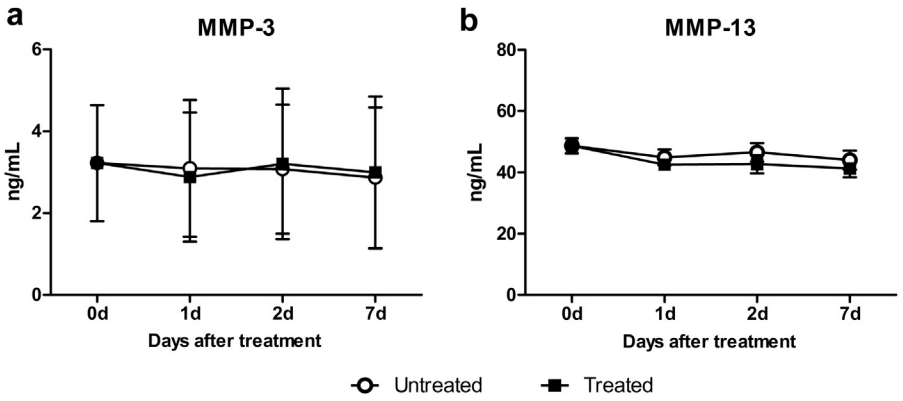

Fig. 7. Release of matrix metalloproteinases 3 (MMP-3) (a) and 13 (MMP-13) (b) into the culture medium after extracorporeal shock wave treatment. No differences were observed between treated and untreated cells at any time point, n = 7.

the exception of treatment of chronic wounds (Schaden et al. 2007), no other clinical applications of soft-focused ESWT have been reported to date.

For the first time, the effects of soft-focused ESWs were analyzed in a primary culture of human tendon cells while adherent to a culture plate. Our rationale was to allow, during ESW treatment, cell-cell contacts and interactions between cells and extracellular matrix, as these interplays represent a crucial point in the mechanotransduction process. Indeed, physical forces influence conformational changes in membrane proteins, such as integrins, which result in intracellular signaling affecting gene expression and release of growth factors (Shyy and Chien 1997; Skutek et al. 2001). For these reasons, our *in vitro* model allows us to overcome the limitation of previous *in vitro* studies in which cells were treated in suspension (Chao et al. 2008; Leone et al. 2012; Vetrano et al. 2011). Because our findings with respect to proliferation and expression of scleraxis and type I collagen were comparable to those reported in these previous studies, the present investigation has to be considered further and strong confirmation of the effect of ESWs on tendon cells; at the same time, it provides a more accurate model for the study of shock wave mechanoresponse in tendon cells. Furthermore, the similarity of the acoustic impedance of the watery medium in the water bath to that of human tissues makes our model even closer to physiologic conditions (Kuhn et al. 2008). A further advantage of the use of soft-focused rather than focused shock waves on adherent cultures is the possibility of treating a larger area of cell culture. Indeed, in our model adherent cell culture, the traditional focused shock waves would have not reached an adequate number of cells, and thus, the total biological effect would have been underestimated. Finally, since we did not use a specific technique to select only tenocytes, we treated a heterogeneous cell population, which better reflects the total cell population of a physiologic tendon.

Table 1. Cumulative nitric oxide production assessed in tendon cell culture medium

	NO release (μM)	
Time	Untreated	ESWT
2 h	Undetected	Undetected
24 h	1.63 ± 1.53	0.96 ± 0.65
48 h	3.87 ± 2.09	1.37 ± 0.99*

ESWT = extracorporeal shock wave treatment. *$p<0.05$, extracorporeal treated cells versus untreated cells, n = 7.

Our findings revealed that shock waves act positively on TC proliferation and viability, at both 7 and 14 d after treatment, although the high interdonor variability did not always allow determination of statistically significant effects. These data are in agreement with some previous data indicating that the effect of ESWT was observed no earlier than 2 wk after shock wave exposure (Speed 2004; Vulpiani et al. 2009).

At the molecular level, the first interesting data concern the expression of scleraxis, a transcription factor specific for tenocytes and their progenitors. This protein, together with the transcription factor NFATc (nuclear factor of activated T cells), regulates the expression of COL1 A1 gene in tendon fibroblasts, and is part of the regulatory network involved in the differentiation of mesenchymal cells into fibroblasts (Lejard et al. 2007). Our data confirm this evidence: after the significant increase in scleraxis expression 1 d after treatment, we observed up-regulation of the COL1 A1 gene 3 d later, although the increase was not significant. Higher levels of SCX and COL1 A1 have been observed in tenocytes derived from pathologic tendons, compared with healthy tenocytes (Leone et al. 2012), and in this study, shock wave treatment provoked a decrease in their molecular levels. These apparently controversial observations confirm that shock waves normalize the metabolic activities of cells.

Changes in total collagen content and composition have been previously reported in tendinopathy, including an increase in the proportions of type III and type V collagen, as well as an increase in the type III/type I collagen ratio (de Mos et al. 2007; Ireland et al. 2001; Lui et al. 2010; Maffulli et al. 2000; Riley et al. 1994). Our data indicated no change either in collagen III or in the collagen III/collagen I ratio after ESWT.

As in other tissues, tendon healing represents a multistep process marked by different phases, based mainly on an inflammatory mechanism (Schulze-Tanzil et al. 2004). Inflammation has recently been reported to play an important role in the onset and maintenance of tendinopathies (Rees et al. 2013). So, because of the dual role of cytokines, in tendon healing and degenerative conditions, for the first time the ESW-mediated release of cytokines by tendon cells was analyzed. These data assume an important implication as they could contribute to better explain the effects and the possible mechanism of ESWs in the treatment of tendinopathy. Production of TNFα was not affected by ESWT, whereas treated TCs released a significantly larger amount of IL-1β with respect to untreated cells, with a peak immediately after treatment (24 h) and a decrease over time in culture. In addition to its important role in normal physiologic events in tendon homeostasis and repair, IL-1β is also a stimulus for the production of MMPs, considered to be responsible for extracellular matrix (ECM) degradation and, consequently, degeneration of the tendon (Archambault et al. 2002; Clegg et al. 2007). For this reason, we also evaluated whether the increase in IL-1β corresponded to the production of MMP-3 and MMP-13, implied in the de-structuring of the bundles of collagen fibers (Archambault et al. 2002; Tsuzaki et al. 2003). Both MMP-3 and MMP-13 were barely detectable after ESWT, suggesting that the increased level of IL-1β released by TCs after ESWT was not correlated with the degradation of ECM. So, in this case, the increase in IL-1β should be interpreted as the primer for the consequent increase in IL-6, which, in turn, promoted the increase in IL-10, which peaked after 48 h. This pathway agrees perfectly with the healing inflammatory mechanism, where the initial acute response is followed, about 48 h after the stimulus, by the production of IL-10, an anti-inflammatory cytokine responsible for the self-resolving phase of inflammation (Schulze-Tanzil et al. 2004). Similarly, TGFβ, a growth factor with anti-inflammatory properties, exhibited a strong increase 48 h after ESWT. Taken together, these results indicate that ESWT was able to induce the initial, beneficial inflammatory phase of the tissue healing mechanism. Experimental models of mechanotransduction have revealed a significant correlation between physical forces and expression of TGFβ and SCX (Maeda et al. 2011). In particular, it has been found that mechanical forces regulate the expression of SCX through activation of a TGFβ/Smad-mediated pathway, which acts to maintain the integrity of the ECM.

Transforming growth factor β is known to be involved in other phases of tissue healing, such as cell proliferation, cell viability and stimulation of collagen production (Zhu and Burgess 2001). Growth factors are among the most important molecules involved in the healing process, but their specific actions are not well characterized. Wang et al. (2005) reported that the transfer of exogenous VEGF gene to proliferating tenocytes increased the expression of endogenous TGFβ. It is likely that these factors work synergistically, playing an integral role in the tendon healing process. However, the role of VEGF in tendon healing is not completely understood. Indeed, although VEGF induction is necessary for tissue repair, it has been also associated with sustained elevation of tendon pathology (Molloy et al. 2003; Pufe et al. 2005). It has been observed that increased levels of VEGF within an injury site are correlated with a well-defined pattern of vascular ingrowth from the epi- and intratendinous blood supply toward the site of repair, providing extrinsic cells, nutrients and growth factors to the injured area (Molloy et al. 2003). On the other hand, there is also evidence that VEGF-induced angiogenesis might be correlated with degenerative tendon disease, inducing expression of MMPs and inhibition of TIMPs (tissue inhibitors of metalloproteinases) (Pufe et al. 2005). One of the main reported effects of ESWT treatment in various tissues (although not yet in tendon tissue) is the neo-angiogenesis promoted by VEGF (Furia et al. 2010; Ito et al. 2009; Mittermayr et al. 2011; Wang 2003; Wang et al. 2003). In our study, ESWT induced a marked increase in VEGF starting 48 h after treatment. This increase can be explained by the fact that IL-6 and IL-10 are the main cytokines stimulating VEGF production (Tartour et al. 2011). Together with the production of inflammatory cytokines and growth factors, such as TGFβ, the significant production of VEGF indicates that ESWT has a profound influence on several aspects of the tendon healing process, including neo-angiogenesis.

In addition to inflammatory cytokines, growth factors and other factors, some cell-to-cell and cell-to-matrix interactions are mediated by some soluble mediators, including NO (Hwang et al. 2000; Schäffer et al. 2002). Contrary to other authors (Chao et al. 2008) who have reported that ESWT promotes the release of NO, we observed significantly lower levels of NO in ESW-treated cells compared with untreated cells. These differences can probably be explained by the different protocol we used; it has been found that the response of cells to shock waves can depend on the type of generator

used, as well as on different energy settings and the type of shock waves produced (Martini et al. 2006). Indeed, in their study Chao et al. (2008) treated rat tenocytes with very high EFD shock waves (0.36 mJ/mm^2) using the traditional cell suspension model. They observed an increase in NO only when cells were treated with a low number of impulses ($\leq$100). The differences in experimental protocol make it difficult to compare our study with that of Chao et al. However, because we observed significant effects of ESWT on TCs, it is possible that these shock wave-related adaptive changes may, at least in part, be controlled by a process in which mediator(s) other than NO play a pivotal role. For example, physical forces could affect the cytoskeleton through mechanotransduction, inducing activation of membrane proteins (integrins and receptors coupled to G-proteins) that could be responsible for the cell responses observed in our study, such as the increase in TGFβ production. Indeed, it is known that this growth factor is able to stimulate migration, proliferation and collagen synthesis in tendon cells (Tsai et al. 2011). It would be important to verify the hypothesis that soft-focused shock waves could activate tendon progenitor cells from their niches, starting from our experimental model. This could prefigure a new rationale for routinely employing soft-focused shock waves to treat the failed healing status that distinguishes tendinopathies.

Acknowledgments—The authors thank Dr. Johannes Holfeld, Department of Cardiac Surgery, Medical University of Innsbruck, Innsbruck, Austria, for his valuable suggestions on the use of the water bath; Massimiliano Marco Corsi-Romanelli, Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy, for his precious contribution to the work; Alessandra Colombini for her precious help in reverse transcription polymerase chain reaction analyses; Gabriele Thiebat, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy, for providing the waste tendon materials; and Jocelyn Serfontein for her precious help in revising the article. The study was partially supported by the Italian Ministry of Health.

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