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EXPERIMENTAL STUDY

Extracorporeal shock wave therapy promotes cell proliferation and collagen synthesis of primary cultured human tenocytes

Mario Vetrano · Federica d'Alessandro · Maria Rosaria Torrisi · Andrea Ferretti · Maria Chiara Vulpiani · Vincenzo Visco

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Abstract
Purpose The aim of this study was to investigate whether the effects of extracorporeal shock wave therapy (ESWT) could affect the behavior of primary cultured human tenocytes over a 12-day period.
Methods In this controlled laboratory study, primary human tenocytes were established from semitendinosus tendons collected from 3 patients undergoing arthroscopic anterior cruciate ligament (ACL) reconstruction. Cell viability, overall cell morphology, cell proliferation, and collagen synthesis following ESWT have been evaluated.
Results ESWT significantly interferes with the overall cell morphology, by impairing dedifferentiation of the cells. Furthermore, a shock wave-mediated growth-promoting effect was measured by the MTT (tetrazolium) colorimetric assay and by the proliferation marker Ki67. Lastly, a significant increase in collagen (mainly type I) synthesis by ESWT-tenocytes compared with control cells was found.
Conclusions Shock wave treatment promoted cell growth and collagen synthesis of primary cultured human tenocytes. The clinical benefits of ESWT may be ascribed to an increased efficiency of tendon repair after injury.

Keywords Tendon healing · Shock waves · Human tenocytes · Collagen · ESWT

Mario Vetrano and Federica d'Alessandro equally contributed to this work.

M. Vetrano · M. C. Vulpiani
Physical Medicine and Rehabilitation Unit, Department of Orthopaedics and Traumatology, "Sapienza" University of Rome, Faculty of Medicine and Psychology, Sant'Andrea Hospital, Via di Grottarossa 1035, Rome, Italy
e-mail: mariovetrano@gmail.com

M. C. Vulpiani
e-mail: mariachiara.vulpiani@gmail.com

F. d'Alessandro · M. R. Torrisi · V. Visco (✉)
Cellular Diagnostic Unit, Department of Clinical and Molecular Medicine, "Sapienza" University of Rome, Faculty of Medicine and Psychology, Sant'Andrea Hospital, Via di Grottarossa 1035, Rome, Italy
e-mail: vincenzo.visco1@uniroma1.it

F. d'Alessandro
e-mail: fe.dale@libero.it

M. R. Torrisi
e-mail: mara.torrisi@uniroma1.it

A. Ferretti
Orthopaedic Unit and Kirk Kilgour Sports Injury Centre, Department of Orthopaedics and Traumatology, "Sapienza" University of Rome, Faculty of Medicine and Psychology, Sant'Andrea Hospital, Via di Grottarossa 1035, Rome, Italy
e-mail: aferretti51@virgilio.it

Introduction

The incidence of tendon injuries has increased during the last years [17], and several studies report that the following healing process is often altered in chronic tendinopathies [20]. It has also been observed that tendon healing can be optimized by different techniques [19], including the extracorporeal shock wave therapy (ESWT) [24].

ESWT has been recently used worldwide in the treatment of different pathologies, with promising results, although our knowledge of the specific mechanisms by which it induces therapeutic effects remains largely limited. Although in clinical practice it has been first used in the management of the recurrent renal stone disease [4], afterward many studies have also tested the efficacy of

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shock wave treatment in soft tissue disorders and musculoskeletal complaints [11, 26–28, 33, 36, 37], in order to prevent the surgical therapy. Studies performed on in vivo animal models have showed a dose-dependent effectiveness after shock wave administration [21, 23, 29], indicating that the different energy devices seem to interfere with the successful response [22].

Though the tenocytes are the major cellular component of the tendon, responsible for its structural integrity by the synthesis and regulation of the abundant but strictly organized extracellular matrix (ECM), they represent only 5% of the normal tissue volume, whereas the remnant 95% is formed by ECM, mainly of type I Collagen [34]. This low cellularity of the tendon justifies the difficulties to establish primary cultures of tenocytes as well as the poor healing tendency of the musculoskeletal tissue [30, 39].

To better elucidate the therapeutical effects of ESWT on chronic tendinopathies, primary cultures of human tenocytes have been established for treating cells with an electromagnetic shock wave generator. In previous reports [27, 33, 36, 37], satisfactory results were obtained treating human tendinopathies with ESWT at energies ranging from 0.08 to 0.40 mJ/mm^2; thus, the same dosages to the primary cultured human tenocytes were applied and their behavior over a 12-day period following the exposure was investigated.

Earlier studies were conducted to assess the shock waves' "short-term" effects using cell lines [9] or animal-derived primary cell cultures [5], meaning that the experiments were mainly performed to measure the biological activities of the cells within few days after the treatment (from 10 min till 7 days). However, this work focused on the evaluation of the "long-term" in vitro burden of different doses of shock wave treatment on tenocytes (meaning that those observations went beyond 12 days after the exposure) because it was previously observed that the therapeutical benefits of ESWT were detected no earlier than 2 weeks following the exposure [27, 33, 36, 37].

Furthermore, according to a recent paper showing the biological effects of pulsed electromagnetic fields (PEMFs) on human tenocytes [7], the tenocytes growth and collagen accumulation of ESW-treated compared with control cells were analyzed.

In fact, because several authors [25, 31] emphasized the fundamental role of cell proliferation and collagen synthesis for the healing process during tendinopathies, those variables were measured in relation to the effects of ESWT on cultured tenocytes. In our hypothesis, an ESWT-induced tendon repair may provide the explication of their clinical efficacy at doses ranging from 0.08 to 0.40 mJ/mm^2.

Hence, the main purpose of this study was to investigate in vitro whether ESWT stimulates tenocyte proliferation and collagen synthesis over a 12-day period following the treatment.

Materials and methods

Tissue samples, human primary cultured tenocytes, and treatments

Three primary cultures of human tenocytes have been established using semitendinosus tendon biopsies obtained from three male patients enrolled in this study (17, 28 and 37 years of age), who underwent arthroscopic anterior cruciate ligament (ACL) reconstruction. The Institutional Review Board of "Sapienza" University and Sant'Andrea Hospital approved the study protocol, and all patients gave their written informed consent to the experimental study.

Tissue biopsies were derived from osteotendinous junction, then cut into small pieces (2.5–3.0 mm^3) and digested with 2 mg/ml collagenase type I (GIBCO). The samples were centrifuged at 1000 rpm for 10 min: the supernatants were discarded [20] and the pellet was cultured in a sterile flask (Falcon), in D-MEM, supplemented with 10% fetal bovine serum (FBS, Hyclone, Euroclone; Europe) and 1% penicillin/streptomycin/glutamine solution (GIBCO).

Primary cultures were named TS1, TS2, and TS3 (TS: Tendon Semitendinosus). They were expanded for two passages (P2) and when they reached 80–85% confluence, at P3, were used for the following experiments. Once confluent growth was achieved, the medium was discarded, and the tenocytes were detached by the addition of one mL of 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA, Euroclone), counted in a counting chamber and used for all experiments.

In order to assess the shock wave effects, at passage P3 primary cultured tenocytes were divided into two groups: ESWT treatment group (connected to the shock wave generator, as previously described [16]) and ESWT non-treatment group as control.

The shock wave treatment was applied using an electromagnetic shock wave generator MODULITH® SLK (STORZ MEDICAL AG; Tägerwilen, Switzerland), whereas the control group was maintained in the same culture conditions, without previous shock wave exposure.

A cryogenic vial (Corning Incorporated, NY, USA) containing a cell suspension with 1×10^6 cells/ml was placed on shock wave generator with a coupling gel (Aquasonic 100; Parker Laboratories, Fairfield, New Jersey, USA) to minimize the loss of shock wave energy at the interface between the head of the device and the cryovial. The tube was placed exactly in the focus of the application pad under ultrasonographic control. The administering

physician was experienced in the use of extracorporeal shock wave therapy in the treatment of various musculoskeletal disorders.

The cells were first treated with a single shock wave exposure at three different doses (0.08, 0.14, and 0.17 mJ/mm^2 either at 500 or at 1000 impulses) and then 0.14 mJ/mm^2 energy level at 1000 impulses was selected. Tenocytes without shock wave stimulation were used as control.

Treated and untreated cells were incubated at 37°C for 12 days and observed at 1, 4, 8, and 12 days. Shock wave treated or untreated human tenocytes were seeded into six-well culture plates (7×10^3 cells/well) (Falcon) and grown in complete medium for 12 days and then assessed for cellular morphology. At 1, 4, 8, and 12 days, cells after ESWT and corresponding controls were observed, and photomicrographs were recorded by an Axiovert S100 inverted microscope (Carl Zeiss, Microlmaging Inc, GmbH, Germany), equipped with a Camera Power Shot A640 (Canon Ōta, Tokyo, Japan). Following previous studies performed in vitro on the differentiation of human skin fibroblasts [1, 15], tenocytes were distinct upon their phenotype into two subpopulations: Elongated (E) and Ovoid (O) cells. Quantitative analysis was performed on a cell count of five photomicrographs for each time point either in treated or in untreated tenocytes.

Cell viability and cell growth assays

ESW-treated and untreated tenocytes were counted in duplicate—using an inverted microscope Axiovert S100 (20×)—immediately before and few minutes after the exposure with a counting chamber, and cell viability was determined by a 0.5% trypan blue exclusion assay (Euroclone).

To assess cell growth, human tenocytes were seeded into six-well culture plate (Falcon) (7×10^3 cells/well) and cultured for 12 days following exposure (or not), as described earlier. The cells were detached daily and counted using a counting chamber. Cell growth was expressed as number of cells/ml, and all assays were performed in duplicate.

According to Widera et al. (*2009*), the population doubling time (*dt*) was calculated using the algorithm provided by http://www.doubling-time.com [38].

Assessment of cell proliferation and morphology by immunofluorescence

Cells were fixed at 1, 4, 8, and 12 days in paraformaldehyde at 4%, treated with 0.1 M glycine and with 0.1% Triton X-100 to allow membrane permeabilization.

Tenocytes were characterized by staining vimentin, a mesenchymal cell marker that labels an intermediate filament of cytoskeleton, with a mouse monoclonal antibody anti-vimentin (1:100) (Clone V9 DAKO, Denmark).

Cells were also incubated alternatively with the following primary antibodies: the rabbit polyclonal antibody anti-Ki67, a nuclear marker of cycling cells (1:50; Zymed Laboratories; San Francisco, CA, USA); the mouse monoclonal antibody anti-collagen type I (1:200; Sigma, Chemicals, St. Louis, Mo., USA), used to ascertain which type of collagen secretion was predominant in cell culture; and the rabbit polyclonal antibody anti-Calreticulin (1:50; Genese Produtos Diagnosticos Ltda Stressgen; Spain), which is a specific marker for endoplasmic reticulum (ER) [12] used to define actively secreting cells. Nuclear staining for total cell counting was performed by the addition of 1 μg/ml of DAPI (4′,6′-diamino-2-phenylindole) (1:10,000) (Sigma, Chemicals, St. Louis, Mo., USA). Monoclonal and polyclonal antibodies were obtained from animals immunized with human antigens, which are specifically recognized by those reagents. The primary antibodies were visualized, using goat anti-mouse IgG-FITC (1:50; Cappel Research Products, Durham, NC, USA) and goat anti-rabbit IgG-Texas Red (1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA).

All the signals were analyzed by recording and merging single-stained images at 40× with an Axiovert 200 inverted microscope, and image analysis was then performed using an Axiovision software.

Ki67 detection was performed on TS1-3 cultures at 1, 4, 8, and 12 days after ESWT by an immunolabeling with anti-Ki67 antibodies. For quantitative analysis, Ki67$^+$ cells were counted on fields randomly taken from five different experiments, and the values have been expressed relative to the corresponding untreated cell values as relative fold expression. For untreated cells, the amount equals 1.0 by definition.

Collagen type I detection was performed on TS2 primary culture at 1, 4, 8, and 12 days after ESWT by an immunolabeling with an anti-Collagen I monoclonal antibody, and the values have been expressed as percentages of Collagen type I expressing cells.

Colorimetric MTT assay

To measure mitochondria cell activity after shock wave stimulation, by the 3-(4,5-dimethylthiazol)-2,5 diphenyl tetrazolium bromide (MTT; Sigma) assay, viable tenocytes from each tube were cultivated in a 24-well plate at 3×10^4 cells/cm^2 (Falcon, Becton–Dickinson; France) over a 12-day period. Briefly, at each time point, cells were incubated with MTT (1 mg/ml) and lysed in dimethyl sulfoxide. Mitochondrial activity was measured by two different MTT assays, first after 24 and 48 h and then after 1, 4, 8, and 12 days following shock wave

exposure. The first experiment (using three different doses, as described) was performed to select the optimal dose of ESWT for the following tests, whereas the second analysis (using a single dose of 0.14 mJ/mm^2 at 1000 impulses) assessed the long-term (up to 12 days) burden of shock wave exposure on primary cultured tenocytes.

The absorbance at 570 nm was measured by a microplate reader (Multiscan Spectrum Thermo Electron Corporation, Vantaa, Finlan). The values are expressed relative to the corresponding untreated cell values as relative fold expression. For untreated cells, the amount equals 1.0 by definition.

Sircol collagen assay to assess total collagen synthesis

The Sircol^{TM} Collagen Assay (Biocolor Ltd., UK) is a dye-binding method planned for the analysis of collagens released in culture medium in vitro.

Cells were seeded at 3×10^4 cells/cm^2 density and incubated for 12 days. After incubation, at 1, 4, 8, and 12 days, 100 µl sample medium was collected and 1.0 ml Sircol Dye Reagent was added. The tubes were centrifuged, then carefully inverted and the supernatants were drained off. The pellets were mixed with 750 µl acid-salt wash reagent, centrifuged and then treated with 250 µl alkali reagent. Two hundred microliters of aliquots of each sample was read with spectrophotometer (Multiscan Spectrum Thermo Electron Corporation) at 555 nm. To quantify total collagen, a standard curve was created using 5, 10, and 15 µl collagen standards [5].

Statistical analysis

Results have been expressed as the means ± standard deviations (SD) from five independent determinations. Significance was calculated using Wilcoxon rank-sum test; values of $P < 0.05$ were considered statistically significant.

Results

Effects of ESWT on cell viability at short time points to select an optimal dose

Energies of 0.08, 0.14, and 0.17 mJ/mm^2 either at 500 or at 1000 impulses applied to the cell cultures did not show any significant effect on the common parameters of cell viability, cytoskeleton organization, and overall cell morphology. These were evaluated by trypan blue exclusion method (scoring >80% of viable cells) and MTT assay (Fig. 1a), by immunofluorescence microscopy after the vimentin intermediate filament labeling (which confirms the mesenchymal origin and excludes possible derangement of the cells) (Fig. 1b) and by phase contrast microscopy analysis, respectively.

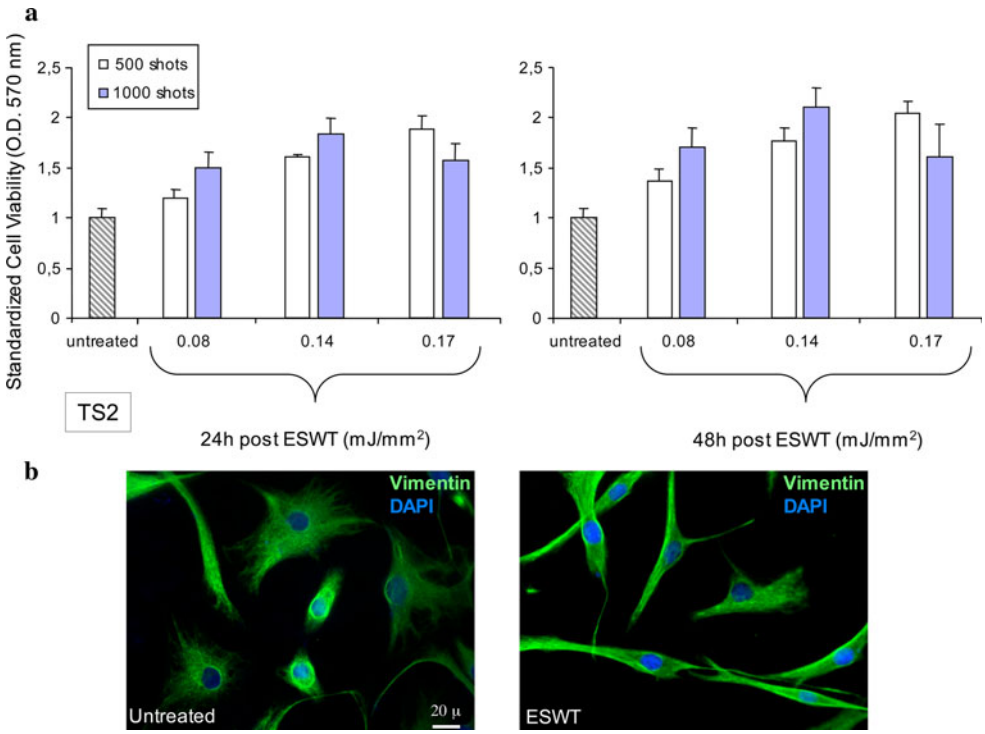

Fig. 1 ESWT short-term effects (24-48 h) on cell viability and cytoskeleton organization of cell culture. **a** MTT assay after 24 and 48 h from ESWT at 0.08, 0.14, and 0.17 mJ/mm^2 (500 and 1000 impulses) on tenocyte cultures. The values for ESW-treated tenocytes are expressed relative to the corresponding untreated cell values as relative fold expression. For untreated cells, the amount equals 1.0 by definition. Results are expressed as mean values ± SD (standard deviations) from five different assays. The absorbance was read at 570 nm. **b** Immunofluorescence analysis achieved on cell culture with anti-vimentin (*green*). Nuclei are stained with DAPI. *Bar* 20 µm

The dose of 0.14 mJ/mm^2 (1000 impulses) was carefully chosen for the following treatments, because it showed clinical efficacy in vivo [26–28, 33, 36, 37] and it did not negatively interfere with the in vitro cell viability (evaluated by MTT assay, as shown in Fig. 1a). At this dosage, either after 24 or after 48 h from the exposure, a weak increase in the number of viable cells was detected in treated tenocytes compared with control cells.

Effects of ESWT on cell morphology at long time points

In this model, the human tendon cells seeded in vitro showed different patterns of cell morphology, as expected [2, 30, 34, 40]. Indeed, inside the cultures, a first group of classically elongated (*E*), *fibroblast-like,* and bipolar tenocytes, as well as a second group of rounded, polygonal, ovoid (*O*), and enlarged cells reminding a *tenoblast-like* morphology were observed (Fig. 2).

Then, a quantitative analysis to check the percentages of E and O morphologies in TS1, TS2, and TS3 cultures found a correlation between the cellular shapes, and the shock wave exposure was performed.

In particular, the analysis conducted after 4 days up to the following 12 days (as documented in Fig. 2 by photomicrographs of representative fields) showed in TS2 and TS3 cultures a phenotypic drift observable in control cells compared with ESWT-tenocytes, whereas in TS1 this effect was significant only in cells cultured on 6-well plates (data not shown). As shown in Fig. 2, shapes and sizes of untreated cells became progressively heterogeneous, which is indicated by the increasing number of O compared with E cells. Conversely, the shock wave exposure provided to prevent the cellular drift. Untreated

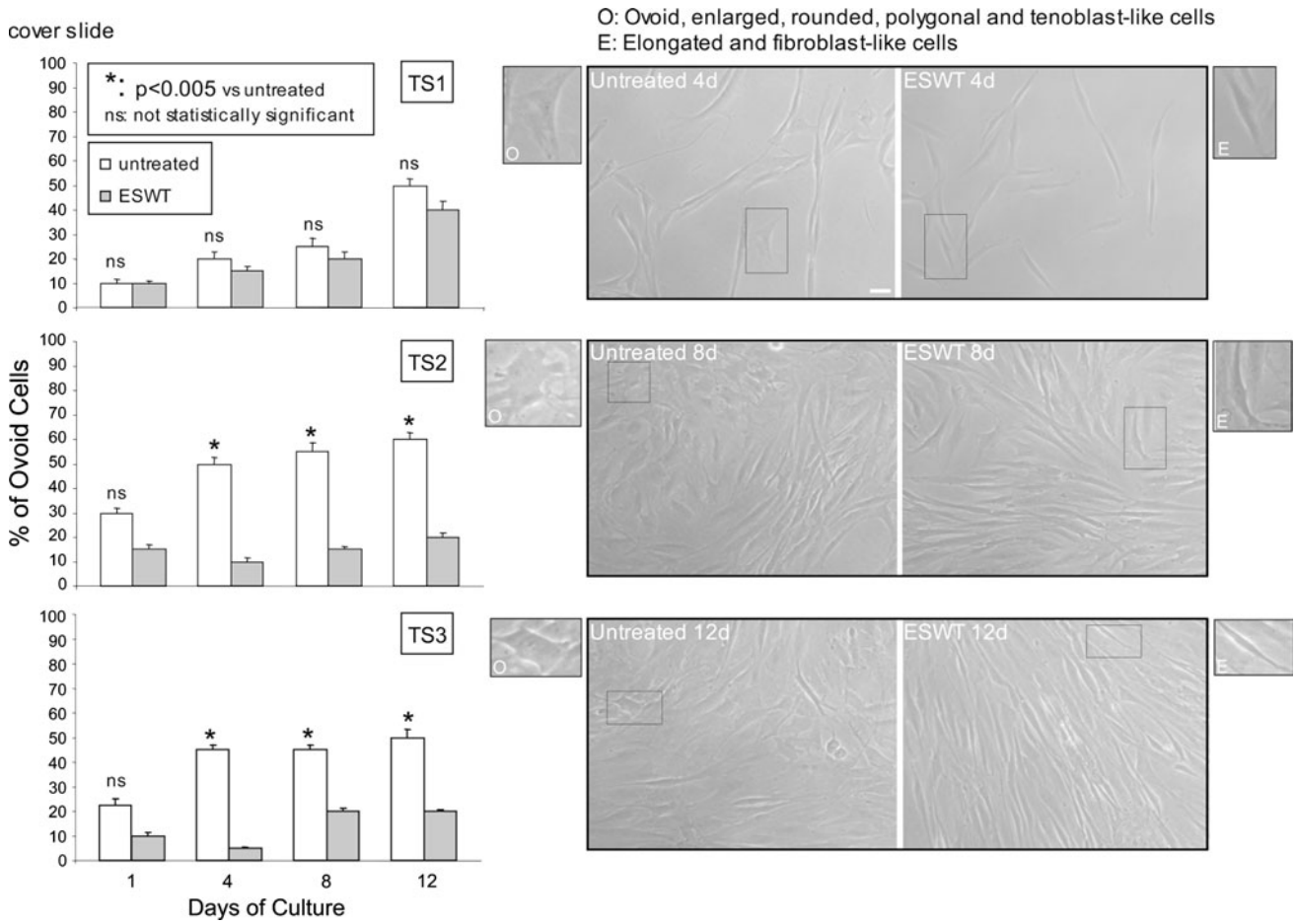

Fig. 2 ESWT long-term effects (1–12 days) on overall cell morphology of TS1-3 cultures. Quantitative analysis of cell morphology performed over a 12-day period in human TS1-3 tenocytes exposed to shock waves (0.14 mJ/mm^2 and 1000 impulses) or *left* untreated as control. According to morphological criteria, cells defined as *O* (ovoid) or *E* (elongated) were counted from five different fields taken randomly. Results were performed by assessing the percentage of ovoid cells and are expressed as mean values ± SD (standard deviations). Student's *t* test was calculated to evaluate significant differences: values of $P < 0.05$ were considered statistically significant. Photomicrographs are representative of each culture. *Bar* 40 μm

cells were almost ubiquitously constituted of E and O phenotypes, whereas the corresponding ESWT-cultures showed a significant prevalence of the E morphology (Fig. 2).

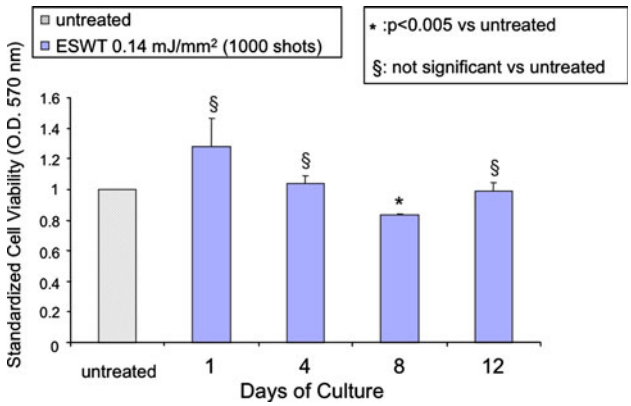

Fig. 3 ESWT long-term effects (1–12 days) on cell viability of cell cultures. MTT assay after 1, 4, 8, and 12 days from ESWT (0.14 mJ/mm^2 and 1000 impulses). Standardized cell viability was expressed as above. The absorbance was read at 570 nm

Effects of ESWT on cell proliferation at long time points

Present results showed that after 1 and 4 days following the shock wave stimulation, cell viability was not significantly modified compared with control (Fig. 3). Interestingly, 8 days after the shock wave treatment, a significant decrease in cell viability was observed, whereas this decline was completely abolished and rebalanced 12 days after the treatment.

Following these data, ESWT and control cells, after 6-well plates seeding, were also counted daily over 12 days, which showed no significant differences between treated and untreated primary cultures.

Quantitative analysis of the percentage of Ki67$^+$ cells revealed an ESWT-mediated proliferative effect on TS1, TS2, and TS3 cultures compared with control. In particular, Ki67 increases became significant up from day 4 on TS1, from day 8 on TS2, and from day 12 on TS3 tenocytes (Fig. 4).

Using the algorithm provided by Widera et al. [38], at day 1, untreated cells compared with ESW-treated cells showed a threefold increase in *dt*, whereas this rate resulted

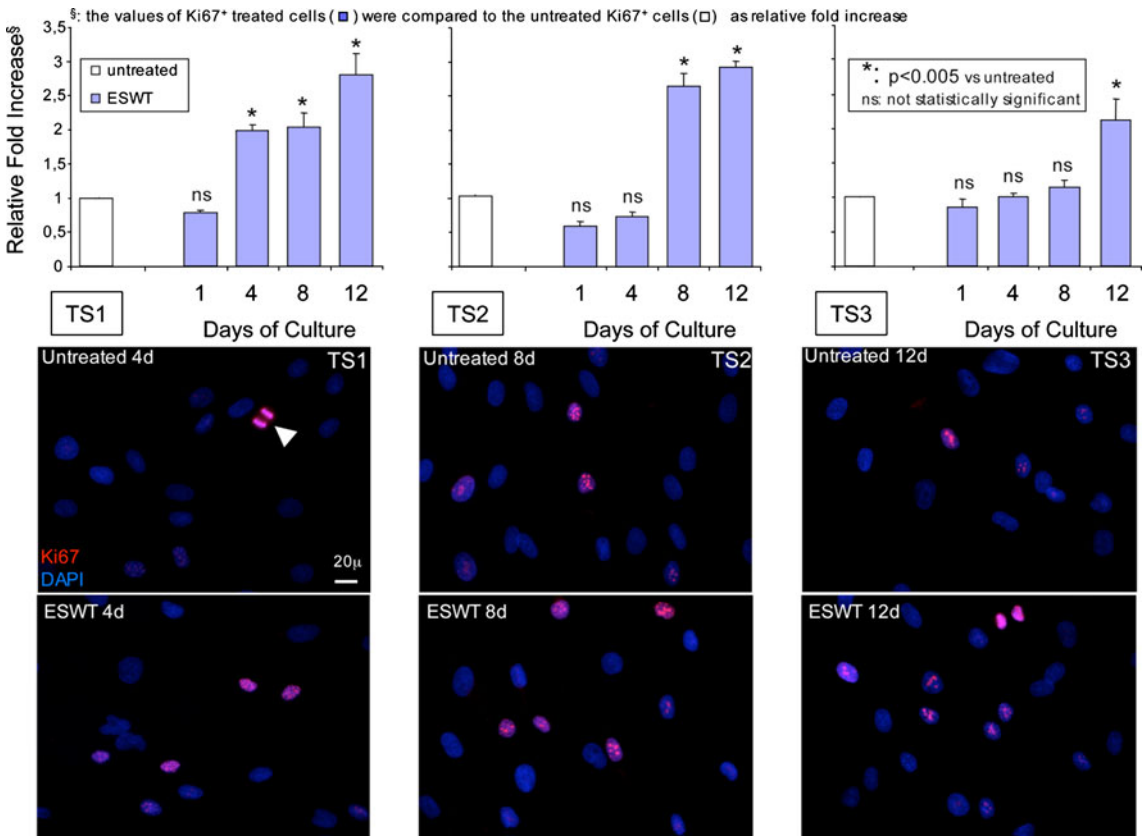

Fig. 4 ESWT long-term effects (1–12 days) on cell proliferation of TS1-3 cultures. Quantitative immunofluorescence analysis of proliferation induced by ESWT (0.14 mJ/mm^2 and 1000 impulses) was performed as above. Immunolabeling with anti-Ki67 (*red*) was achieved on TS1-3 cultures at 1, 4, 8, and 12 d (days) after ESW exposure. Nuclei are stained with DAPI. Photomicrographs are representative of each culture. *Bar* 20 µm

completely inverted at day 12, displaying a double *dt* in ESWT-tenocytes compared with control cells.

Effects of ESWT on the synthesis of total collagen

In vitro collagen production was strongly stimulated by ESWT on TS1-3 cultures. This increase was significant beside control in the all selected times (day 1, 4, 8, and 12, after the treatment) in TS1 and TS2, whereas in TS3 exclusively at day 12 (Fig. 5).

The Collagen I distribution in the cytoplasm was detected by immunofluorescence analysis, showing a perinuclear cell staining localized preferentially in the endoplasmic reticulum, as expected [13] (Fig. 6a). As illustrated in Fig. 6b, the Collagen I^+ cells were then counted and again it was observed that ESWT significantly increased the percentages of Collagen I-secreting cells compared with control, after 8 up to 12 days.

Furthermore, the collagen secretive activity of tenocytes was confirmed by a double immunofluorescence assay showing the intracellular reticular staining of Calreticulin and the Collagen I cytoplasmic staining. In the majority (data not shown) of Collagen I-secreting cells, an overlapping staining with the ER marker Calreticulin was detected in the endomembrane compartment (Fig. 6c).

Interestingly, the secretive activity of cultured tenocytes, indicated by a double positivity for Calreticulin and Collagen I, was mainly confined to those cells presenting the classical elongated tenocyte morphology. In fact, the counting of double positive cells showed that around 75% of them expressed an elongated phenotype (Fig. 6c).

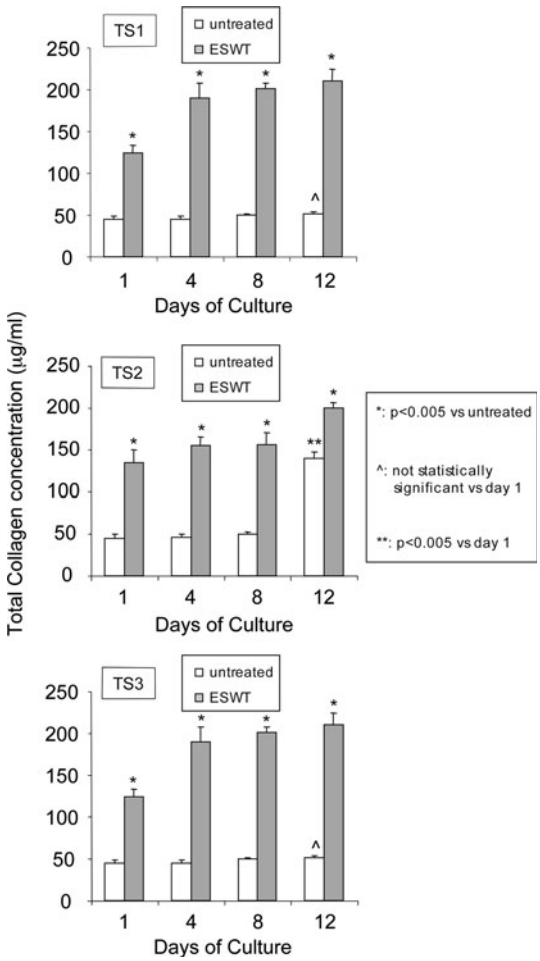

Fig. 5 ESWT long-term effects (1–12 days) on total collagen synthesis by TS1-3 cultures. Collagen quantitation (Sircol Assay) on TS1-3 supernatants collected from cultures at 1, 4, 8, and 12 days after ESW exposure. Amounts of total collagen (ranging from 44.5 to 210) are expressed as µg/ml, and results are expressed as mean values ± SD (standard deviations) as above. Values of $P < 0.05$ were considered statistically significant

Discussion

The principal findings of this work provide a possible explication of ESW-mediated therapeutical benefits in patients affected by chronic tendinopathies. In fact, the biological mechanisms underlying the clinical effectiveness of ESWT in musculoskeletal disorders have not yet been defined so far and their elucidation might hold important clues for ameliorating tendon repair strategies.

In order to ascertain whether the clinical benefits of shock wave treatment in the field of orthopedics can be effectively ascribed to the promotion of an enhanced tissue regeneration and healing processes after tendon injuries (as emphasized by previous report) [3, 5, 14, 24, 25, 32], in this paper, for the first time, the ESWT-mediated effects on primary cultured human tenocytes derived from semitendinosus tendon were analyzed.

Primary cultures of human tenocytes were obtained from explants of semitendinosus tendons, harvested during anterior cruciate ligament (ACL) reconstructions, because it was assumed that the biological activities of such cells should be extrapolated to any other tendon-derived cells.

An energy flux density of 0.14 mJ/mm^2 was applied to the cultures, primarily considering that it showed a clinical efficacy in vivo [26–28, 33, 36, 37].

In addition, the long-term effects of the shock waves exposure on human tenocytes were assessed, because previous remarks revealed that the therapeutical benefits of ESWT were observed no earlier than 2 weeks following shock wave exposure [27, 33, 36, 37]. On the contrary, several studies focused on the early behavior of the cells after the treatment, mainly using cell lines or animal-derived primary cultures [5, 9, 14, 33].

First, the biological significance of a well-known morphological heterogeneity, occurring during tenocyte culture and probably imputable to the trigger of a dedifferentiation

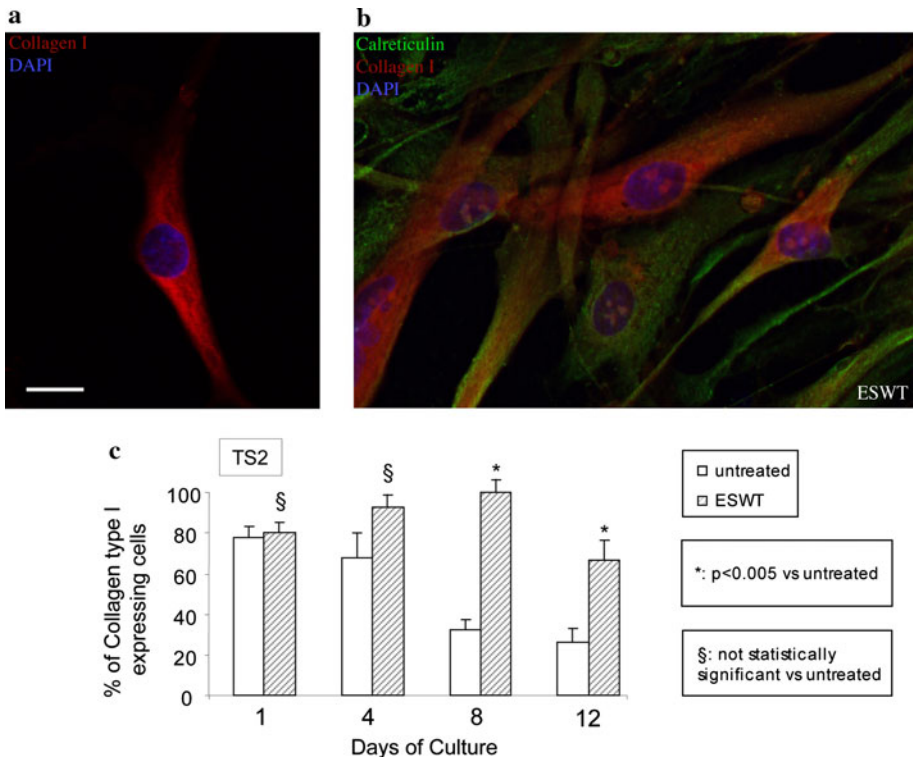

Fig. 6 ESWT long-term effects (1–12 days) on type I collagen synthesis by cultured tenocytes. **a** Immunofluorescence analysis performed on cell culture with anti-type I collagen (*red*), showing a perinuclear cell staining. Nuclei are stained with DAPI. *Bar* 20 μm. **b** Quantitative immunofluorescence analysis performed by assessing the percentage of Collagen type I-expressing cells 1–12 days after ESWT. Results are expressed as mean values ± SD (standard deviations) as above. **c** Double immunofluorescence analysis performed on ESW-treated cells with anti-Calreticulin (*green*) and anti-type I collagen (*red*). Overlapping staining (*yellow*) was detected in some cells. *Bar* 20 μm

program, was investigated [2, 30, 34, 40]. It was consistent with the present data showing either a prevalent, elongated, *fibroblast-like* phenotype, corresponding to the differentiated tenocytes or an ovoid, polygonal, enlarged, *tenoblast-like* morphology. These advisements support the view that untreated primary cultured tenocytes can be mainly considered as differentiated cells [2, 18], although with unstable phenotype and a continuous tendency to dedifferentiate in vitro [34, 40]. In this model, such natural tendency to progressively dedifferentiate was significantly impaired by the shock wave exposure.

Furthermore, morphological changes in tenocytes were previously associated with tendon pathologies, indicating that drifting phenotypes [40] may be responsible for the altered matrix components seen in tendinopathic tendons [8, 20].

In agreement with previous reports [2, 18, 32, 34], in this model, the synthetic activity of the tendon cells, measured over a 12-day period, was mostly ascribable to the classical tenocytes showing the elongated morphology.

In this study, it is shown that ESWT significantly and progressively increased the total collagen synthesis in primary cultures, confirming the shock wave-promoting activities remarked by the morphological data. Interestingly, the main product synthesis by the cultured tenocytes belonged to type I collagen (similarly to Bernard-Beaubois et al. [2]), which represents the major component of the fiber system, possessing high tensile strength and stiffness and which is crucial during the later events of healing, such as tendon consolidation following injuries [3, 32]. This result was not surprising, because the primary cultured tenocytes used in such model were derived from healthy tendons and it is well documented that Collagen I is the major component of normal tendons, whereas cells from injured tendons produce greater amounts of type III collagen [6, 10, 20]. Consistently these elements are characterized by a well-developed and prominent endoplasmic reticulum, confirming the morphological data are mostly characterized by an elongated phenotype, amenable to the differentiated tenocyte. Mechanistically shock wave exposure may prevent the tenocyte dedifferentiation, induce an increased synthetic activity and probably contribute to tendon repair.

In order to assess the shock wave burden on viability and proliferative activity of cultured tenocytes, MTT and immunofluorescence assays have been performed at long time points ranging from 1 to 12 days after the exposure.

MTT revealed that the early behavior of the cells was not significantly modified, whereas after 8 days a weak but significant decrease in the viability was observed. Interestingly, after 12 days, this long-term effect again became not significant. Previous reports could provide a possible explication of those data, showing a delayed increase (after 8 days of culture) in the proliferative activity of shock wave–treated and survived cells [3].

However, to better clarify this result, a daily cell count was performed, measuring the doubling time of the cultures and then labeling the Ki67 proliferation markers

using immunofluorescence, as reported [35]. In particular, the Ki67 staining that identifies the cycling cells showed a significant increase in the number of proliferating tenocytes (in TS1, TS2, and TS3) at long time points, after 4 up to 12 days following the ESWT. Again this result supports the relevance of shock wave exposure in promoting functional activities of tenocytes, such as proliferation, which is considered crucial for the following injury tendon repair.

These results show that, in a model of human tenocytes, shock wave treatment promoted cell growth and synthetic activity, suggesting that the clinical benefits and the timing of this therapy may be explained by an increased effectiveness of the healing mechanisms.

The major limitation of this study includes the application of shock wave therapy on tenocytes derived exclusively from healthy tendons. Therefore, further researches could be useful to clarify whether tenocytes derived from pathological tendons may display a different functional behavior following ESWT. In addition, the present data have been collected on cultures of human tenocytes and required more evidences in vivo.

Conclusion

This study seems to indicate that differentiated tenocytes are metabolically "activated" by ESWT and significantly induced to proliferate. They synthesize in vitro increasing amounts of total collagen (mainly type I) compared with untreated cells, which in contrast enter in a dedifferentiation program, characterized by heterogeneous cell morphology and lower secretive activity. Furthermore, in the management of human chronic tendinopathies, ESWT is highly recommended at doses ranging from 0.08 and 0.17 mJ/mm^2.

Conflict of interest All authors certify they not have signed any agreement with a commercial interest related to this study, and they declare no conflict of interest related to this work.

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