Ultrasound in Med. & Biol., Vol. 40, No. 6, pp. 1204–1215, 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

Laura de Girolamo,* Deborah Stanco,* Emanuela Galliera,†‡ Marco Viganò,* Arianna Barbara Lovati,§ Monica Gioia Marazzi,‖ Pietro Romeo,¶ and Valerio Sansone‖¶

*Orthopaedic Biotechnology Laboratory, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy; †Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, Università degli Studi di Milano, Milan, Italy; ‡IRCCS Istituto Ortopedico Galeazzi, Milan, Italy; §Cell and Tissue Engineering Laboratory, Gruppo Ospedaliero San Donato Foundation, Milan, Italy; ‖Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy; and ¶Orthopaedic Department, Istituto Ortopedico Galeazzi, Milan, Italy

(*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).

Address correspondence to: Laura de Girolamo, Orthopaedic Biotechnology Laboratory, IRCCS Istituto Ortopedico Galeazzi, Via R. Galeazzi 4, 20161 Milan, Italy. E-mail: laura.degirolamo@grupposandonato.it

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