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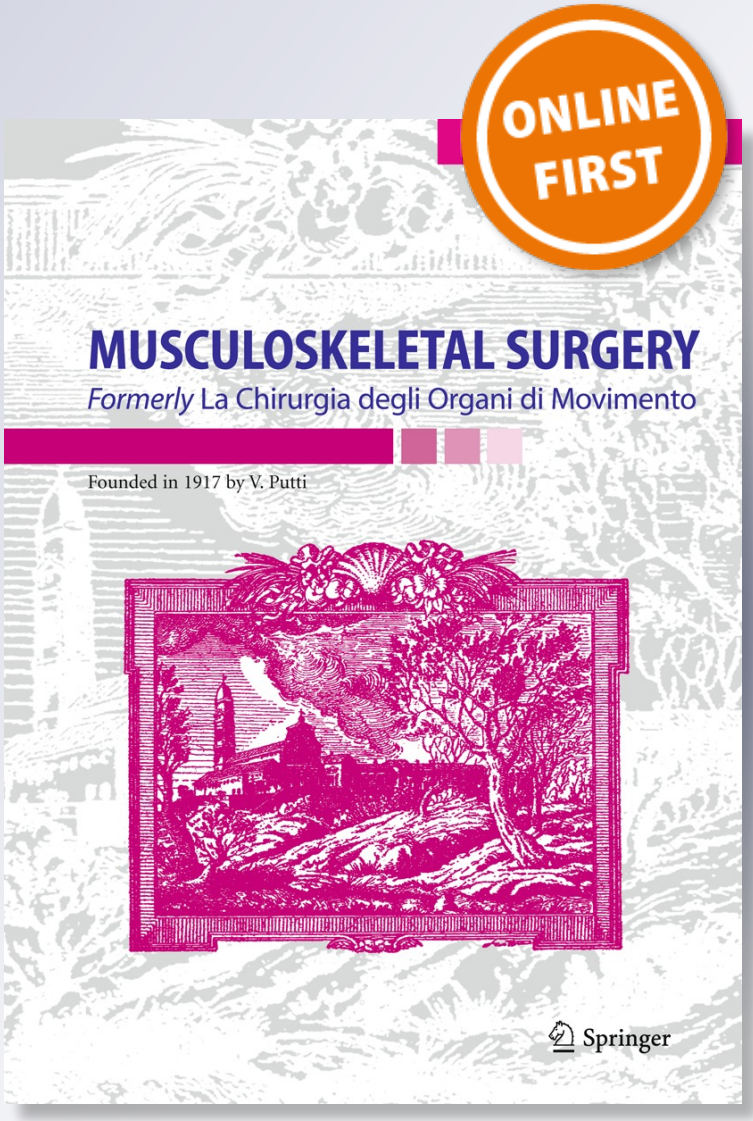

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ORIGINAL ARTICLE

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Abstract Radial shock waves therapy (RSWT) differs from extracorporeal shock waves therapy (ESWT) in that it produces a non-focused wave that is dissipated radially at the skin. Few studies have yet explored the effects of RSWT on bone tissue. Osteoblasts in culture flasks were studied by polymerase chain reaction after treatment with RSW (500 impulses, 0.05 mJ/mm^2). An inhibited osteoblastogenesis was observed, with a statistically significant reduction in type 1 collagen, osterix, bone sialoprotein and receptor activator NF kappa ligand expression at 24 and 48 h, of osteocalcin at 24, 48 and 72 h, and osteopontin at 48 and 72 h. These findings show that RSWT is not indicated for treatment of delayed fracture union, pseudoarthrosis, and complex regional pain syndrome. The observed reduction in the receptor activator of nuclear factor-kB ligand/osteoprotegerin ratio suggests that it has an inhibiting effect on osteoclastogenesis, which could make it a useful tool for applications in proliferative diseases.

Keywords Osteoblast · Osteoclastogenesis · Radial shock waves therapy

Angela Notarnicola and Roberto Tamma contributed equally to the work.

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Abbreviations

RW	Extracorporeal radial pressure waves
PCR	Polymerase Chain Reaction
ESWT, SWT	Extracorporeal shock wave therapy
OBs	Osteoblasts
BMSCs	Bone Mesenchymal Stem Cells
Cbfa1/Runx2	Core binding factor A1/Runt-related transcription factor 2
COLL I	Collagen type I
OSTC	Osteocalcin
OPN	Osteopontin
BSPII	Bone sialoprotein II
RANK	Receptor activator of nuclear factor-kB
RANKL	RANK ligand
BMSCs	Bone marrow stromal cells
OPG	Osteoprotegerin
PBS	Phosphate-buffered saline
α-MEM	α-Minimal essential medium
FBS	Fetal bovine serum
PTH	Parathyroid hormone
EFD	Energy Flux Density
TGF-ß1	Transforming growth factor-beta 1
IL-10	Interleukin 10
TNF-alpha	Tumor Necrosis Factor-alpha
VEGF	Vascular endothelial growth factor

Background

The application of radial shock waves therapy (RSWT) is a recent technique that has been employed since the year 2000 to treat painful muscle–tendon syndromes. The ballistic source consists of a compressor-free ballistic cylinder that fires a projectile against a metal applicator placed on

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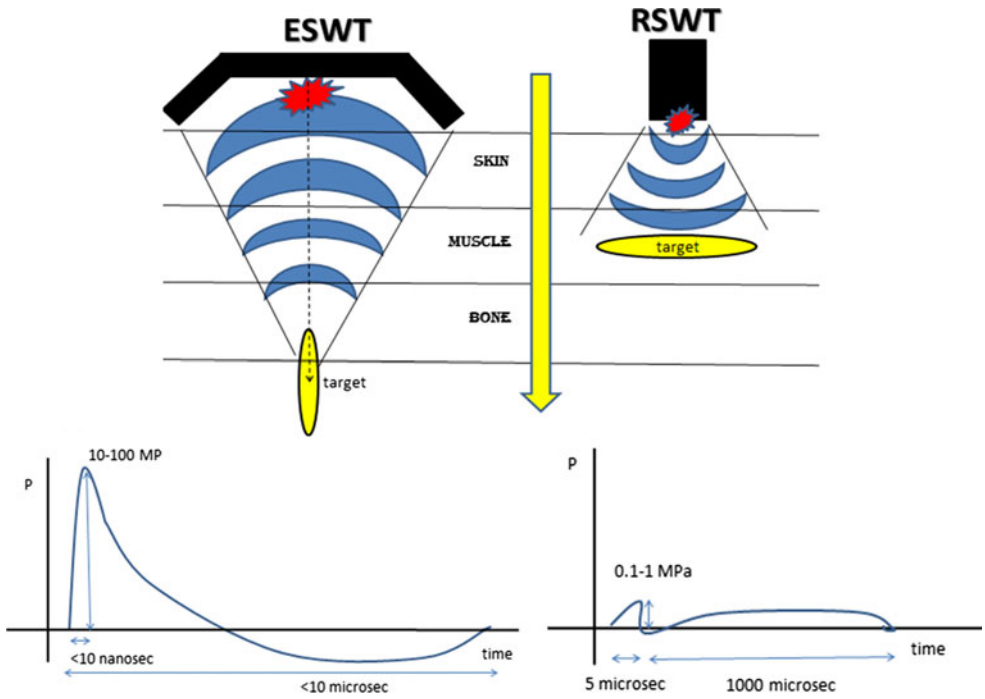

Fig. 1 Physical characteristics and wave propagation of extracorporeal shock wave therapy (*ESWT*) and radial shock wave therapy (*RSWT*)

the patient's skin [1]. The projectile generates stimulation waves in the applicator head, which are transmitted as pressure waves to the underlying tissue. The acoustic wave propagates radially and has a low penetration power (less than 3 cm) and force of impact (0.02–0.06 mJ/mm^2) [1]. The radial wave sends a positive pressure impulse that reaches a maximum value of 0.1–1 MPa within a few milliseconds and then decreases to environmental pressure values [2]. The time taken for the radial wave to rise, that is about one microsecond, is too long, and so, the curve of the concave surface of the ray is too wide for it to be possible to focus it, so the term "to focus" is not used for radial waves, unlike for extracorporeal shock waves therapy (ESWT) [1] (Fig. 1). The main reported effect is on modulating nociception, related to its biological actions [3–5].

Aim of the present work is to explore the effects produced by radial pressure wave therapy on the osteoblasts. Bone mass maintenance is well known to be the result of the balance of two processes: osteoformation induced by the osteoblasts (OBs) and resorption induced by the osteoclasts (OCs) [6]. Osteoblastogenesis causes the differentiation of bone mesenchymal stem cells (BMSCs) into functional OBs, involving the activation of different transcription factors such as the core binding factor A1/runt-related transcription factor 2 (Cbfa1/Runx2), which directly regulates the expression of the major OB markers such as collagen type I (COLL I), osteopontin (OPN), bone sialoprotein II (BSPII) and osteocalcin (OSTC) [7]. Osteoclastogenesis is regulated by a signaling pathway that involves both the receptor activator of nuclear factor-kB (RANK) expressed on the surface of mature osteoclasts and their precursors, and the RANK ligand (RANKL) expressed on the bone marrow stromal cells (BMSCs) [8]. In addition, osteoprotegerin (OPG), a soluble decoy receptor secreted by osteoblasts (OBs) and BMSCs, competes with RANK for binding to RANKL, resulting in an anti-osteoclastogenic effect [9]. In this work, we have explored the effects of RSWT on these bone tissue pathways, which have never previously been studied.

Methods

The study was approved by the local university ethics committee and by the local institutional animal care and use committee.

Murine calvaria osteoblasts (OBs)

The frontal and parietal bones were removed from 5- to 6-day-old mice (c57bl/6j) in sterile conditions, and the periosteum was detached using scissors. The calvaria fragments were digested with 0.5 mg/ml Clostridium histolyticum neutral collagenase (Sigma Chemical Co, St. Louis, MO, USA) in phosphate-buffered saline (PBS) at 37 °C for 60 min. After digestion, calvaria fragments were washed vigorously three times with α-minimal essential medium (α-MEM), then transferred to 12.5-cm^2 cell culture flasks and cultured in α-MEM supplemented with 10 % fetal bovine serum (FBS, Gibco, Uxbridge,UK), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 50 IU/ml mycostatin (Gibco, Uxbridge, UK) at

37 °C, in a water-saturated atmosphere containing 5 % CO_2. The medium was changed every 3 days. Under these conditions, the osteoblasts (OBs) in the fragments proliferated and migrated to the culture surface, reaching confluence within 2 weeks. Cells were then trypsinized and transferred to appropriate dishes for characterization and experiments.

Osteoblasts (OBs) characterization

Murine osteoblasts were characterized according to the well-established parameters of alkaline phosphatase activity, the production of cAMP in response to 10^{-8} M of PTH (Parathyroid hormone; Sigma Chemical Co, St. Louis, MO, USA), and synthesis of osteocalcin in response to 10^{-8} M of 1,25-dihydroxyvitamin D3 (Sigma Chemical Co, St. Louis, MO, USA).

Application of radial shock waves therapy

Calvaria osteoblasts were seeded in 25 cm^2 at a density of $8 \times 10^3/cm^2$ and cultured at 37 °C in a water-saturated atmosphere containing 5 % CO_2. A compressor-free RSWT system was employed (enPuls, Zimmer Medizin-Systeme GmbH, Germany). We applied an energy flux density (EFD) of 0.05 mJ/mm^2.

Some osteoblast flasks were treated with 500 impulses of RSWT at an energy density of 0.05 mJ/mm^2 and a 5 Hz pulse repetition rate, docked by means of a water-filled cylinder, whereas the other flasks were used as controls. A common ultrasound gel was used as contact medium between the cylinder and flask. After RSWT, the culture medium was changed, and the flasks were maintained in an incubator under standard conditions prior to use in the different experiments.

RNA extraction and reverse transcriptase reaction

Osteoblasts were subjected to RNA extraction using spin columns (RNAeasy mini Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with the AccuScript™ High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). A 10-μl aliquot of the initial mix [1 μg RNA, 1 mM dNTPs, 50 pmol Oligo(dT), DEPC H_2O] was incubated at 65 °C for 5 min and on ice for 1 min, and 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, and 1 μl (40 units) of RNaseOUT were then added. After 2 min of incubation at 42 °C, 1 μl (50 units) of SuperScript II RT was added and incubation at 42 °C was resumed for 50 min and then at 70 °C for 15 min. Then, 1 μl (2 units) of Rnase-H was added, and a further 20 min of incubation at 37 °C were done to complete the reaction.

Table 1 Primers sequence from 5′–3′

OPG/S	AGTCTGAGGAAGACCATGAG
OPG/A	AAACAGCCCAGTGACCATTC
GAPDH/S	TGCGACTTCAACAGCAACTC
GAPDH/A	CTTGCTCAGTGTCCTTGCTG
RUNX2/S	CGTCAGCATCCTATCAGTTC
RUNX2/A	CCGTCAGCGTCAACACCATC
OSTEOCALCIN/S	TCTCTGACCTCACAGATCCC
OSTEOCALCIN/AS	CCTTATTGCCCTCCTGCTTG
COLLAGEN1/S	GGCTCCTGCTCCTCTTAG
COLLAGEN1/AS	ACAGTCCAGTTCTTCATTGC
OSTEOPONTIN/S	ATCTCAGAAGCAGCCTCTCC
OSTEOPONTIN/AS	ATGGTCATCATCGTCGTCC
BSP/S	AGCAGCACCGTTGAGTATGG
BSP/AS	TTCTGACCCTCGTAGCCTTC
RANKL/S	GCTCCGAGCTGGTGAAGAAA
RANKL/AS	CCCCAAAGTACGTCGCATCT
OSX/S	TATGCTCCGACCTCCTCAAC
OSX/AS	AATAGGATTGGGAAGCAGAAAG

A adenine, *C* cytosine, *G* guanine, *T* thymine, *S* sense, *AS* antisense

Real time polymerase chain reaction (PCR)

cDNA was amplified with the iTaq SYBR Green supermix with the ROX kit (Bio-Rad Laboratories), and PCR amplification was obtained using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

Messenger RNA expression for OPG, RANK-L, RUNX2, OPN, BSP, OSTEOCALCIN, Type 1 COLLAGEN and GAPDH as the housekeeping gene, was evaluated by real-time PCR. The primer sequences, all with a 60 °C annealing temperature (Operon Biotechnologies GmbH, Cologne, Germany), are reported in the Table 1.

The amplification process includes three steps:

1. Incubation at 95 °C for 3 min;
2. Incubation at 95 °C for 15 s;
3. Annealing and extension at 60 °C for 30 s.

Steps 2 and 3 were repeated 40 times.

After the last cycle, melting curves analyses were performed on the 55–95 °C interval in increments of 0.5 °C. The fold change values were calculated by the Pfaffl method [10].

Preparation of cellular extracts

No sign of cellular distress was revealed at optical microscopy. The mice calvaria osteoblasts were lysed with RIPA ice cold buffer (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 % NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 8 μg/ml leupeptin)

added with 1 mM sodium orthovanadate for 10 min. Consecutive extracts were centrifuged at 14,000 revolutions per minute (rpm) for 15 min at 4 °C to separate the nuclei, while the supernatant was harvested for protein dosage. The protein extract concentrations were determined by the BCA Protein assay Reagent Kit (Pierce Biotechnology, Inc. Rockford, IL).

Statistical analyses

Statistical analyses were performed by Student's t test for the comparison of continuous variables using the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL). Statistical significance was set at P values of <0.05.

Results

RUNX2

RUNX2 is a key regulator of the osteoblast-specific gene expression involved in the secretion of bone matrix, as COLL1 and OPN [11]. The results of the experiment did not reveal significant variations in the RUNX2 mRNA at 24, 48, and 72 h after RSWT as compared to unstimulated controls (Fig. 2).

Osterix

Osterix is a zinc finger–containing transcription factor that is essential for osteoblast differentiation and bone formation [12]. A significant reduction in Osterix mRNA expression was observed at 24 and 48 h after RSWT as compared to the controls. By 72 h, the values had returned to the same levels as the unstimulated controls (Fig. 3).

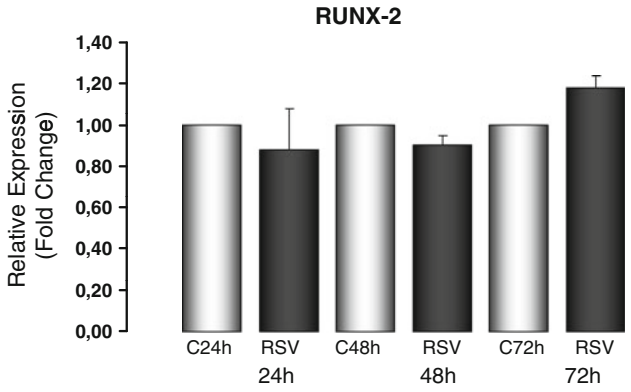

Fig. 2 Real-time PCR for the RUNX2 transcription factor. No variation in the RNA expression was observed after radial waves stimulation, as compared to unstimulated controls (*C* control, *RSV* radial waves)

Osteocalcin

Osteocalcin is a vitamin K-dependent non-collagenous bone matrix protein. Osteocalcin is synthesized by osteoblasts and is a well-known marker of the viability, differentiation, and osteogenic ability of these cells [13]. At the three assessment times (24, 48, and 72 h), RT-PCR showed significant reductions in osteocalcin mRNA expression as compared to unstimulated osteoblasts (Fig. 4).

Type I collagen and osteopontin

Type I collagen and Osteopontin are the most abundant proteins in the bone extracellular matrix [14]. RSWT treatment induced a statistically significant reduction in collagen type I mRNA already at 24 h, which lasted until 48 h after stimulation and then expression rose higher than in controls by 72 h (Fig. 5). Osteopontin messenger RNA expression showed a later reduction than collagen I, as shown by the statistically significant reduction in osteopontin messenger RNA expression at 48 and 72 h as compared to unstimulated controls (Fig. 6).

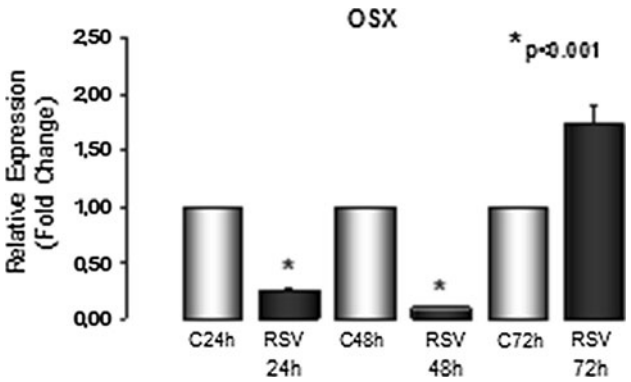

Fig. 3 RT-PCR showed a reduced Osterix protein RNA expression at 24 and 48 h after radial waves stimulation as compared to unstimulated controls (*C* control, *RSV* radial waves)

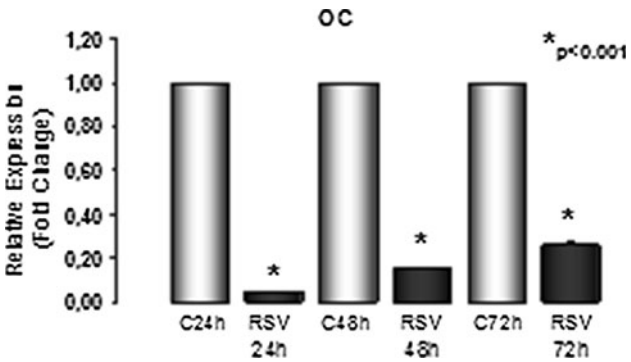

Fig. 4 RT-PCR showed a reduction in osteocalcin mRNA expression at 24, 48, and 72 h after RW stimulation; osteocalcin is a specific late marker of osteoblast differentiation (*C* control, *RSV* radial waves)

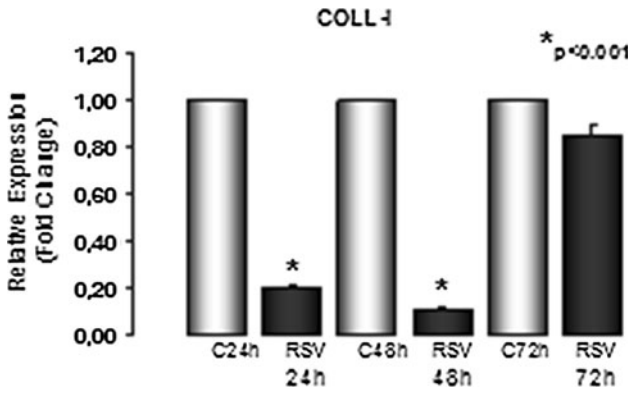

Fig. 5 Effects of radial wave application on bone tissue extracellular matrix protein expression. RT-PCR showed a reduced Collagen I protein RNA at 24 and 48 h after stimulation (*C* control, *RSV* radial waves)

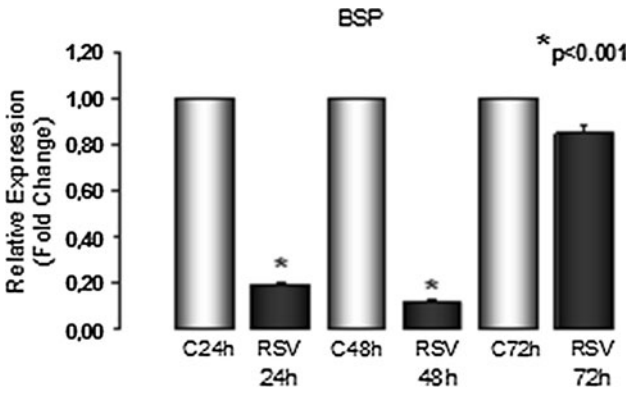

Fig. 7 Real-time PCR for bone sialoprotein, a bone tissue extracellular matrix protein. At 24 and 48 h after RW application, a reduced RNA expression was observed as compared to unstimulated controls (*C* control, *RSV* radial waves)

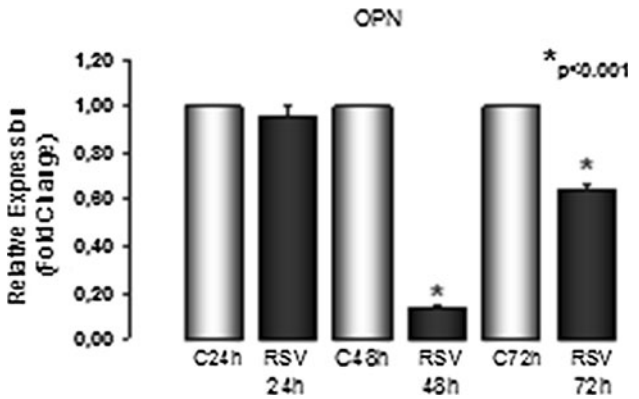

Fig. 6 At 48 and 72 h after radial wave application, osteopontin RNA expression was reduced; osteopontin is a bone tissue extracellular matrix protein (*C* control, *RSV* radial waves)

Bone sialoprotein (BSP)

Bone sialoprotein (BSP), a major constituent of bone matrix, is found almost exclusively in mineralized tissues and is therefore considered a potential marker of bone metabolism [15]. Radial shock waves treatment also affected BSP expression, inducing a statistically significant reduction in messenger RNA expression at 24 and 48 h after stimulation and recovery by 72 h (Fig. 7).

RANKL and OPG

Osteoclasts are the cells responsible for bone matrix degradation, and their formation is regulated by osteoblasts via the secretion of the cytokines involved, namely RANKL and OPG. RANKL promotes osteoclast maturation and activation. OPG is the decoy receptor for RANKL that blocks RANKL action and thus inhibits osteoclast differentiation and function. It is therefore the balance between RANKL and OPG expression that determines the extent of osteoclast activity and subsequent bone resorption [16].

RT-PCR assessment of OPG and RANKL expression after RSWT stimulation did not elicit any significant variation in OPG, whereas there was a statistically significant reduction in RANKL expression after 24 and 48 h (Fig. 8). The reduced RANKL/OPG ratio suggests an osteoblast-mediated inhibition of osteoclastogenesis.

Discussion

In clinical practice, radial shock waves therapy has been employed for several years now in the management of painful muscle–tendon diseases. Greve et al. [17] supported the combined application of radial waves with sural and plantar fascia stretching exercises. The authors obtained efficacious, durable results in patients unlikely to respond to physiotherapy alone, with bilateral chronic plantar fasciitis showing an ultrasound thickness of 4–9 mm [18]. In a previous experience, Cacchio et al. [19] had presented a randomized clinical trial of patients affected by calcific tendinitis of the shoulder. RSWT was shown to improve the clinical picture, significantly reducing the tendon calcifications in 86 % of the treated patients, as compared to 13 % of controls. The clinical improvement persisted at 6 months after treatment.

Few works have yet investigated the effects of RSWT on bone tissue. In clinical applications, no bleeding effect on bone tissue has been demonstrated [20]. Byron et al. [21] made a radiographic and scintigraphic study of bone tissue undergoing radial pressure waves and found no metabolic activity. Microstructural examinations and assessment of the cortical elasticity also failed to show significant variations after RSWT [22]. In an application, in the horse for navicular disease, Brown et al. [23] found that

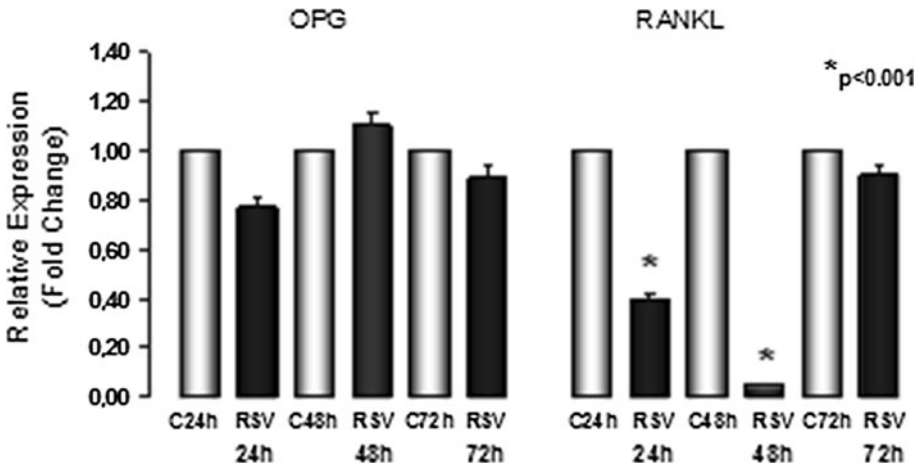

Fig. 8 Effects of radial waves on the RANKL/OPG system expression pattern in osteoblast cultures. mRNA expression was assessed by real-time PCR. While no variations were observed for OPG, a reduced RANKL expression was found at 24 and 48 h after stimulation, responsible for a reduction in the RANKL/OPG ratio (*C* control, *RSV* radial waves)

the clinical benefit was limited to the analgesic effects of the treatment and saw no signs of stimulation of the bone tissue. No modulation of the nociceptive system was observed at the level of the periosteum, and the clinical effects were attributed to modulation at the skin level, where an increase in substance P and calcitonin gene-related peptide was demonstrated [24].

In the literature, the need to gain a better understanding of the biological effects of radial shock waves treatment, in order to define the best clinical indications and application protocols, has been underlined. In the treatment of chondrocytes, an increased membrane permeability and cellular vitality has been reported [25, 26]. However, evaluations of the effects on the cellular metabolism did not demonstrate any induction of nitric oxide (NO) or prostaglandin E2 (PGE2) synthesis nor release of glycosaminoglycans (GAG). Moreover, after 48 h from treatment, a reduction in GAG synthesis was reported [26]. The main reported result of RSWT is in modulating pain. Yamashita et al. [27] observed a short-term increase in endorphin synthesis, together with a long-term suppression of inflammatory processes at the level of the dorsal ganglia neurons. Sugioka et al. [28] described an inhibition of the production of proinflammatory algogenic cytokines in tenocytes treated with radial waves.

In this experience, we observed a prevalently inhibitory effect of radial shock waves on the specific proliferation and metabolic activation genes, as compared to unstimulated controls. A statistically significant reduction in the expression of Coll-1, Osterix, BSP, and RANKL (after 24 and 48 h), OC (after 24, 48, and 72 h), OPN (after 48 and 72 h) was elicited, whereas no variations were found in RUNX2 and OPG expression.

As suggested in the literature we, too, found that radial shock waves do not have a metabolic activation effect on the bone tissue [17, 19–24]. In fact, although we observed a late response for RUNX2 and Osterix, there was a persistent inhibitory effect on the expression of the other messenger RNA. For this reason, radial shock waves therapy does not seem indicated in the treatment of skeletal tissues when a metabolic activation effect is required, such as in complex regional pain syndrome, delayed union, and pseudoarthrosis [20–23]. In our view, further studies are warranted to see what effects are produced by radial shock waves Therapy protocols with different EFD and numbers of impulses. It should also be remembered that this treatment is strongly limited by dissipation of the pressure wave already at the skin level, so it does not reach bone segments lying deeper than 2.5 cm^2.

Conclusions

The reduction in the RANKL/OPG ratio suggests that RSWT has an inhibiting effect on osteoclastogenesis. Further studies are needed to examine the effect of RSWT in proliferative diseases, such as Paget's disease, in which the underlying pathogenetic mechanism is a hyperactivity of OBs and OCs. In such conditions, radial shockwaves therapy may be indicated because of its inhibitory action on both osteoclastogenesis and osteoblastogenesis.

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Conflict of interest The authors declare that they have no competing interests.

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